EXHIBIT A

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Gene Transfer into Mammalian Somatic Cells *In Vivo*

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ABSTRACT: Direct gene transfer into mammalian somatic tissues *in vivo* is a developing technology with potential application for human gene therapy. During the past 2 years, extensive progress and numerous breakthroughs have been made in this area of research. Genetically engineered retroviral vectors have been used successfully to infect live animals, effecting foreign gene expression in liver, blood vessels, and mammary tissues. Recombinant adenovirus and herpes simplex virus vectors have been utilized effectively for *in vivo* gene transfer into lung and brain tissues, respectively. Direct injection or particle bombardment of DNA has been demonstrated to provide a physical means for *in situ* gene transfer, while carrier-mediated DNA delivery techniques have been extended to target specific organs for gene expression. These technological developments in conjunction with the initiation of the NIH human gene therapy trials have marked a milestone in developing new medical treatments for various genetic diseases and cancer. Various *in vivo* gene transfer techniques should also provide new tools for basic research in molecular and developmental genetics.

KEY WORDS: gene transfer, *in vivo*, gene therapy, transfection, semantic.

I. INTRODUCTION

Gene therapy is becoming increasingly accepted as a likely approach for treatment of various and specific genetic diseases.[1–10] The current strategy for this approach is to first identify the mutant gene(s) causing a genetic defect, then to supplement the defective somatic tissues with the correct functional gene(s). Recombinant DNA technology has been used to identify and to isolate specific genes that are responsible for certain genetic diseases. Versatile technologies must now be developed to transfer functional genes into a wide variety of somatic tissues to effect gene therapy.

Gene transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transduced cells into host target organs is the main strategy for human gene therapy. Alternatively, direct transfer of functionally active foreign genes into mammalian somatic tissues or organs *in vivo* is another attractive strategy for gene therapy. During the past 2 years, a number of methods have been reported to achieve these goals with varying degrees of success. Some of these methods were obtained serendipitously. For example, direct injection of plasmid DNA into mouse muscle tissues resulted in expression of marker genes for an indefinite length of time.[11,12]

Recombinant retroviral vectors optimized for transgene expression were employed to transduce hepatocytes *ex vivo* that were later transplanted, via intrasplenic injection, into the liver of autologous host animals, effecting gene expression *in vivo*. With this method, significant levels of the low-density lipoprotein receptor gene were detected in serum of test animals.[13] Retroviral vectors have also been reported to be effective for *in vivo* and *in situ* infection of blood vessel tissues.[14] Portal vein and direct injection of retrovirus preparations into liver tissue were also shown to effect gene transfer and expression *in vivo*.[15–17] More recently, intratracheal infusion of recombinant adenovirus into lung tissues was

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found to be highly effective for *in vivo* transfer and prolonged expression of foreign genes in lung respiratory epithelium.[18] Herpes simplex virus vectors were also used successfully for *in vivo* gene transfer into brain tissue.[19,20]

In addition to various vector-mediated gene transfer methods, progress has also been made on direct (nonviral) gene transfer technology. We and others have demonstrated that many different types of somatic tissues can be effectively engineered *in vivo* with various marker genes constructed as plasmid DNA via a particle bombardment method.[21-23] Other techniques developed for this purpose include polylysine-mediated transfer of DNA into hepatic cells,[24,25] lipofectin- or liposome-mediated gene transfer to tissues of internal organs,[14,26,27] vesicle complexes as vehicles for gene delivery into liver,[28] electroporation of skin tissues *in vivo*,[29] and others.[11,30,31] Virtually all of these developments in gene transfer technology have the same ultimate goal: to establish and optimize techniques and procedures for future application to human gene therapy.

Besides the technological advances in gene transfer in experimental animals, even more important progress was made in the past 2 years in clinical trials of pilot human gene therapy studies. After going through extensive reviews of gene transfer and clinical treatment protocols, two challenging clinical gene therapy trials began in September 1990. In the first trial, two patients suffering from adenosine deaminase (ADA) deficiency (also known as severe combined immune deficiency (SCID) syndrome) were treated with ADA gene-transduced peripheral blood lymphocyte cells.[9,32] In another case, two melanoma patients were treated with tumor-infiltrating lymphocytes (TIL) transduced with the tumor necrosis factor (TNF) gene.[10,33] These trials, conducted by W. F. Anderson, R. M. Blaese, S. A. Rosenberg and their colleagues, were the result of many years of experimentation to improve *in vitro* gene transfer techniques in primary cultures of various lymphocyte systems. Based on their earlier studies in clinical trials using TIL cells modified to express the marker gene NPT-II for neomycin resistance (Neo)[34], they demonstrated that all tested patients tolerated the treatment well, and they observed no side effect from the gene transfer procedure per se. Although the efficacy of the present gene therapy treatments for ADA deficiency and melanoma patients has yet to be determined, an early review of the two ADA-treated patients revealed dramatic improvement.[32] These NIH clinical trials have thus set the stage for a new era of medical treatment of human diseases using gene therapy.

Only a few years ago, the concept of gene therapy for human genetic diseases, cancer, or other maladies was often viewed as a last resort. A key concern was that the tools and methods for gene delivery were limited, but today various breakthroughs involving *in vivo* and *ex vivo* gene transfer technology have been realized. This, in conjunction with the initiation of the Human Genome Project and with the increasing number of new genes identified for human diseases and cellular physiology, have confirmed the legitimacy and urgency of systematic research and development of gene therapy-related technologies.

Several new, drastically different gene transfer methods were developed very recently, almost simultaneously. Most of these methods were technically designed for specific organs. Therefore, there has been little time for adaptation or extension of specific techniques to other experimental systems, either by the group that invented the method or by other laboratories. As a result, at this stage, one cannot make direct or effective comparisons of gene transfer and expression efficiencies. This review is thus made episodic rather than systematic. An effort was also made to assess advantages and disadvantages of certain methods for future application to human gene therapy.

II. VIRUS-MEDIATED GENE TRANSFER *IN VIVO*

A. Retrovirus-Mediated Gene Transfer *Ex Vivo* and Implantation of Transduced Cells into Animals

Retrovirus vectors for mammalian gene transfer have been studied extensively during the last decade. A key development in applications of this technology to human gene therapy has been the implementation of maximal safety mea-

sures in the construction of amphotropic, replication-deficient retrovirus systems. This aspect, and other safety considerations, of using retroviral vectors for human gene therapy have been reviewed extensively by Temin.[35]

Techniques and protocols for using retroviral vectors in the transfer of genes into mammalian cells have been reviewed previously by many investigators, including Eglitis and Anderson,[36] Kohn et al.,[37] Friedmann,[38,39] Karson,[40] Kriegler,[41] Temin,[35] Miller,[42] Williams,[43] and Lo et al.[44] Continuous progress is being made on generating replication-deficient and highly "disarmed" virus vectors and amphotropic virus strains, efficient packaging cell lines, increased titers of virus preparations, and improved viral infectivity.

Using retrovirus vectors, three leading groups at the National Institutes of Health have pioneered the research on human gene therapy in various areas. Anderson, Blaese, Culver, and their co-workers[32] have introduced a normal (or correct) gene encoding adenosine deaminase (ADA) into peripheral blood lymphocytes (PBL) derived from ADA-deficient patients. These *ex vivo*, viral-transduced PBL cells were enriched in primary cultures by selection (Neo), then later injected into donor patients. Rosenberg, Anderson, Chiang, and their co-workers[33,34] have also used retroviral vectors to transfer the NPT-II gene and a potentially useful therapeutic gene (tumor necrosis factor or TNF) into tumor-infiltrating lymphocyte (TIL) cells that were isolated from tumors of melanoma patients. In January 1991, researchers transfused the *ex vivo* propagated TIL cells containing the TNF gene into two cancer donor patients, so that these cells may infiltrate the patients' tumors and release TNF to destroy cancer cells. Both of these pilot clinical trials are still in progress, so it will be some time before their efficacy can be evaluated fully.

In addition to peripheral lymphocytes and TIL cells, implantation of recombinant somatic cells back into animals, followed by expression of the introduced genes *in vivo*, has also been carried out in several other somatic cell systems. Morgan et al.[45] and Teumer et al.[46] transferred human growth hormone (hGH) genes into human kerotinocytes in culture, then grafted stably transduced epidermal cells in epithelial sheets onto athymic mice. The hGH proteins were detected in the serum of test mice at physiological concentrations for more than 4 weeks.[45]

Ponder et al.[47] isolated recombinant hepatocytes from the liver of transgenic mice and transplanted them via intrasplenic injection into recipient mice. They determined that a large fraction of these cells was localized within the liver parenchyma tissue, but not within the spleen, at 2 months after transplantation. X-gal staining assays showed that the frequency of donor cells transduced with the *E. coli LacZ* gene (encoding for β-galactosidase, β-gal) was between 0.1 and 0.3% of all hepatocytes in the recipient liver tissue, a very appreciable level. High levels of human α1-antitrypsin (α1AT, another transgenic product) were detected in the serum of transplant recipients, with expression being stable for more than 6 months. Their results suggest that transplanted hepatocytes reestablished *in vivo* may survive indefinitely. Ledley et al.[48] and Shen et al.[49] had demonstrated previously that hepatocytes in primary cultures could be transduced using retrovirus vectors, although efficiencies were relatively low in these primary cultures when compared with established cell lines. This relatively low gene transfer efficiency, however, has been improved recently by better cell manipulation techniques for primary cultures of hepatocytes (S. Woo, personal communication). Ponder et al.[50] also reported that lipofectin can be used effectively to deliver genes into primary cultures of hepatocytes, obtaining high levels of transient gene expression in transduced hepatocytes.

More recently, Chowdhury et al.[51] effectively transduced *ex vivo* rabbit hepatocytes in primary culture with recombinant retroviruses carrying a low-density lipoprotein receptor (LDLR) gene. They showed that by performing a 30% partial hepatectomy followed by intrasplenic injection of autologous, transduced hepatocytes, the great majority (95%) of the infused cells were immediately seeded into the liver via the portal circulation. They further demonstrated that upon expression of the LDLR transgene in transplanted hepatocytes, a 30 to 50% decrease in total serum cholesterol in LDLR-deficient rabbits was observed that persisted for the duration of the experiment (4 months). These results pro-

vide a good model system for the systematic exploration of liver-directed gene therapy.

Based on the results of these various groups,[47-51] a combination of more effective methods for hepatic gene transfer and an efficient procedure for transplanting transduced hepatocytes into liver tissue has been developed, which will soon be tested in human hepatic gene therapy. In mid-1991, the NIH Human Gene Therapy Subcommittee approved a proposal by Ledley, Woo, and co-workers[52] to test a gene therapy protocol for human clinical trials in acute hepatic failure and targeting marker genes to patients' hepatic cells. Later, the committee approved a similar protocol by Wilson and co-workers[51,53] to treat familial hypercholesterolemia patients, using an LDLR gene.

Salminen et al.[54] have used retroviral infection to transfer a human multidrug resistance gene (MDR1) into the genome of a rat muscle cell line (L6) and into primary rat myocytes. Transgenic myocytes were implanted into the tibialis anterior muscle of Wistar rats. Expression of MDR1 mRNA in muscles was detected at 5 d after implantation, but was minimal at 12 d. Immunosuppression of the rats with cyclosporine A resulted in detection of MDR1 mRNA at 3 to 4 weeks after implantation, a similar expression pattern to that observed for myoblasts implanted in the muscle of nude mice. These results show that implantation of recombinant myocytes into skeletal muscle may be a useful strategy for gene therapy in certain muscle diseases.

Using a recombinant retroviral vector, Dhawan et al.[55] effectively transduced a myoblast cell line (C2C12) with hGH and *LacZ* genes. After genetically engineered myoblasts were injected into mouse muscle, hGH was detected in serum for up to 3 months. Studies on cellular localization of transgenic β-gal activity showed that transduced, transplanted myoblasts effectively fused with preexisting multinucleated myofibers of target muscle tissues. Using the same approach, Barr et al.[56] reported very similar findings with C2C12 myoblast cells transduced by a calcium phosphate/plasmid DNA coprecipitation method. In this study, the levels of hGH detected in the serum of host mice were significantly lower (~4-fold less) than those observed by Dhawan et al.,[55] presumably due to lower levels of hGH expression in stably transduced myoblast cells.

B. Site-Specific G ne Transfer *In Vivo* Via Retroviral Vectors

To direct tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue-specific can be used. Alternatively, this can be achieved via *in situ* delivery of DNA or viral vectors to specific anatomic sites *in vivo*. Recently, several different techniques were developed for this purpose.

1. Transduction of Arterial Wall Tissues

Nabel et al.[57] first demonstrated that foreign gene transfer to blood vessels *in vivo* could be achieved by implanting *in vitro*-transduced endothelial cells in chosen sites on the arterial wall. However, since this method required cell lines to be established before gene transfer, its application to human somatic gene therapy would be limited. This concern led to the later development of a site-specific *in vivo* gene transfer method for arterial walls.

Using a recombinant retrovirus vector, Nabel et al.[14] reported a catheter delivery system that provided efficient *in vivo* retrovirus infection of specifically chosen arterial wall segments, and thereby effected long-term, site-specific gene expression in pig blood vessel tissues. To introduce either a murine amphotropic retroviral vector or DNA entrapped in liposomes, they inserted a catheter into an arterial segment. The proximal and distal balloons of the catheter were then inflated, generating a closed space into which *LacZ* transduced retrovirus was injected via the catheter device. Polybrene was injected after instillation of the virus to improve infection efficiency. X-gal chromogen assays revealed that extensive β-gal expression was readily detected for a prolonged period of time (10 d to 4 weeks) following the viral infection (Plate 1).* Maximal expression was observed between 2 and 3 months following infection. Also, *in vivo* delivery using liposomes entrapping *LacZ* DNA was found to confer β-gal

* Plates 1 and 2 appear after page 342.

expression; high levels of X-gal staining were detected after 4 d (Figure 1) and the expression persisted for more than 6 weeks.

Nabel et al.[14] have obtained additional data that show that (1) both retrovirus and liposome methods confer gene transfer and expression of marker genes in all tissue layers of the arterial wall, including the intima, media and adventia; (2) various cell types, including endothelium, vascular smooth muscle, etc., are similarly susceptible to both gene transfer methods; (3) expression via either retrovirus or liposomes is limited to the site of gene delivery and is not detectable in liver, lung, kidney or spleen; and (4) no helper virus is present in serum or tested organs.

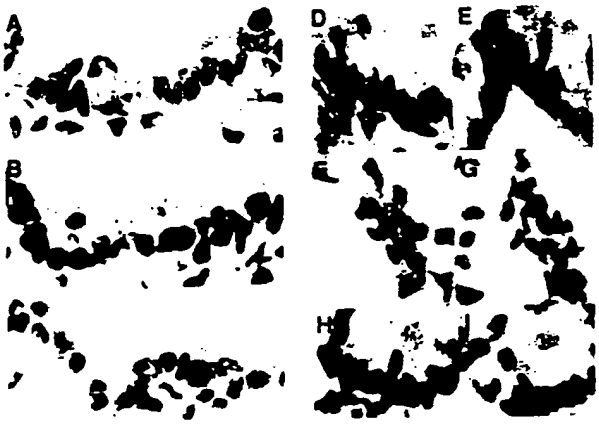

FIGURE 1. *In situ* hybridization evaluation with ^{35}S-labeled Ad-α1AT RNA probes of lung from cotton rats infected *in vivo* with Ad-α1AT by instillation into the trachea. (A) Uninfected lung (PBS control) with antisense probe. (B through I) Several examples of Ad-α1AT-infected lung. (B) Antisense probe. (C) As in (B) but with sense probe. (D) Antisense probe. (E) As in (D) but with sense probe. (F) Antisense probe. (G) As in (F) but with sense probe. (H) Antisense probe. (I) As in (H) but with sense probe. (Reprinted by permission of the publisher from Rosenfeld et al., *Science*, American Association for Advancement of Science, Washington, D.C., 1991.)

These results are important because the catheter device enables site-specific transduction of chosen blood vessel segments. Even more important, because vascular endothelial cells are exposed to blood circulation, this should be an excellent method for the release of transgenic proteins into the bloodstream, delivering secretable therapeutic proteins into the whole body. More recently, Lim et al.[58] also adopted the catheter technique for use in dogs. They showed that, by using this device to deliver the lipofectin-DNA complex, low-level firefly luciferase (Lux) gene expression was detected in arteries. Together, the findings of Nabel et al.[14] and Lim et al.[58] suggest that the balloon catheter method for gene transfer may provide a useful clinical method for treatment of human diseases such as atherosclerosis or certain cancers.

It should not be overlooked, however, that the efficiency of retroviral infection and the resulting gene expression levels are relatively low. On average, about 1% of treated cells are found to be transduced, with up to 10% transduction frequencies occasionally observed (G. Nabel, personal communication). Further optimization of the transduction protocol may result in higher levels of expression. In terms of the biology of the blood vessel tissue *in vivo*, the observed gene transfer efficiency may already be high. This is because blood vessel tissues, consisting of endothelial, smooth muscle, and other cell types, are highly differentiated and as such are not known to actively proliferate or turn over under normal physiological conditions. Since retrovirus infection and viral gene integration into the host cell genome have been shown to occur only in actively dividing cells,[59] to significantly increase *in vivo* infectivity one may have to introduce physical damage or chemical wounding to the target sites of blood vessels. Researchers also need to exercise caution in using X-gal staining for detection of transgenic β-gal activity in mammalian tissue explants, because background and β-gal-like activities can be detected among different tissue samples and may vary among different experimental animals (Yang et al., unpublished data). Flugelman et al.[60] recently showed that this concern may present a problem for X-gal staining of blood vessel tissue. Such concerns can be eliminated for some systems (either quan-

titatively or qualitatively) by using appropriate controls and enzyme staining procedures.

2. Hepatic Gen Transfer

Replication-incompetent retrovirus was used to infect livers of fetal rats by intraperitoneal injection of animals *in utero*, and to infect adult animals by direct injection into the portal vein after partial hepatectomy. With this approach, Hatzoglou et al.[15] showed that the introduced provirus sequences were integrated into the hepatic genomic DNA, with reporter gene expression detectable for up to 8 months postinfection. A liver-specific promoter from a phosphoenolpyruvate carboxykinase (PEPCK) gene was used to drive the NPT-II or bovine growth hormone (bGH) genes.

Successful viral gene expression in these experiments was obtained with the rat liver regeneration system after partial hepatectomy. Physiological and anatomical studies by Higgins and Anderson,[61] Fabrikant,[62] and Grisham[63] showed that hepatocytes can actively divide in the regenerating rat liver following the removal of up to 66% of the liver. Within the first 24 h following partial hepatectomy, synchronized DNA synthesis begins in hepatic cells followed closely by mitosis. The percentage of hepatocytes undergoing DNA synthesis increases from less than 1% to about 50% by 24 h.[62,63] Within 1 month, the liver has returned to its prehepatectomy mass, i.e., a 3- to 4-fold increase in tissue mass. Since Miller et al.[59] demonstrated that only cells that are actively dividing during infection are transduced by retrovirus, the high proliferative activity of hepatocytes in regenerating liver may thus provide an excellent environment for *in vivo* retrovirus infection. Indeed, by infecting hepatic cells of regenerating liver tissues, Hatzoglou et al.[15] demonstrated high levels (15 to 28 ng/ml/2 d) of bovine growth hormone in serum from rats infected with PbGH virus. Furthermore, they showed that the expression of the PEPCK-bGH gene was influenced by diet and hormones in a pattern similar to the endogenous regulation of the PEPCK gene in rat liver. Molecular results demonstrated the integration of foreign genes as provirus sequences in the host cell genome.

By using a different route for administration of virus particles, Kaleko et al.[16] recently reported that *in vivo* gene transfer into mouse liver can be achieved by direct injection of retrovirus preparations into liver parenchyma tissue. Instead of using partial hepatectomy to stimulate cell proliferative activity in the liver, they showed that injection of mice with an appropriate dosage of carbon tetrachloride (CCl_4), 2 d prior to surgery and virus injection, was effective in stimulating mitotic activity in liver parenchyma tissue and resulted in effective retroviral infection. PCR analysis of liver genomic DNA indicated that the presence of NPT-II gene sequences in infected liver was sustained from 10 weeks to 6 months after gene transfer, whereas livers that had not been treated with CCl_4 contained no NPT-II sequences. Two months after viral gene transfer NPT-II enzyme activity was still detectable in transduced liver tissues. These enzyme levels were very low unless the animals had been injected with azacytidine and CCl_4 prior to sacrifice.

Based on an estimate of signal intensity in their PCR analysis, Kaleko suggested that approximately 1 copy of the NPT-II gene per 160 diploid genomes was present in the transgenic liver tissues.[16] If one assumes that, on the average, one copy of the NPT-II gene is incorporated into each hepatocyte, this copy number ratio suggests that about 0.7% (1/160) of the liver's hepatocytes were transduced long term via this protocol. This presumed gene transfer rate should be verified by assaying marker gene expression at the cellular level (e.g., X-gal staining of β-gal transduced cells in liver tissue sections). This information would be very important for future applications of this or other techniques to hepatic gene therapy, because severalfold differences (e.g., 0.7 vs. 5%) in gene transfer efficiency may drastically affect the practicality of employing certain therapeutic genes against target diseases. Taking this viewpoint into consideration, Ferry et al.[17] established a surgical procedure in which regenerating rat liver (after partial hepatectomy) was temporarily excluded from the circulation system and infected *in vivo* by a 10-min asanguineous perfusion with recombinant retrovirus. Using transgenic *LacZ* with nuclear localization signals, they determined that up to 5% of the hepatocytes in the remnant liver lobes expressed

β-gal for at least 3 months after gene transfer. A 5% level of long-term transgene expression in regenerated liver tissue is quite encouraging; this technique apparently represents the most efficient method reported so far for liver-directed gene transfer.[15,16,24,51] To further evaluate hepatic gene therapy technology as a whole, it may be important to compare the various hepatic gene transfer techniques developed by Kaleko et al.,[16] Hatzoglou et al.,[15] Ferry et al.,[17] and Wu et al.[24] (to be discussed subsequently). A direct comparison within the same experimental conditions (e.g., using the *LacZ* gene driven by a strong promoter) may reveal the relative gene transfer efficiencies and the population distributions of transduced cells in target liver tissues.

3. Transduction of Mammary Tissues

In vivo gene transfer using retrovirus vectors has also been demonstrated recently to work in the rat mammary gland system. Wang et al.[64] used a thin needle to inject, via the central duct, recombinant retrovirus into the luminal space of rat mammary glands. One to 2 weeks after injection, β-gal activity was detected in about 0.3% of the total mammary epithelial cells, with expression lasting several more weeks. Southern blot analysis showed that some mammary epithelial cells had integrated the marker gene into the host cell genome, and that these cells were apparently responsible for the low but significant level of marker gene expression *in vivo*. Their results suggest that the mammary gland of breast tissues may also be employed as a target site for future gene therapy.

C. Adenovirus for *In Vivo* Gene Transfer into Lung

One limitation of the retrovirus gene delivery system is that it requires target cell proliferation for gene transfer.[59] However, most organs in adult mammals are fully developed, and most of their cells are terminally differentiated and incapable of active proliferation.[18] Therefore, under normal physiological conditions, it was believed to be difficult to transfer foreign genes *in vivo* to various mammalian somatic tissues via retroviral infection.[18] However, Nabel's[14] results in the vascular system indicate that this perception may need to be reevaluated.

To bypass this potential problem, Rosenfeld et al.[18] used a recombinant adenovirus vector to transfer a recombinant human gene to the lung respiratory epithelium *in vivo*. This approach took advantage of two past observations: " (1) host cell proliferation is not required for expression of adenovirus proteins[65,66] and (2) adenovirus are normally tropic for the respiratory epithelium." [67] In their study, the adenovirus (Ad) major late promoter was used to drive a recombinant α1AT gene and was engineered into an infectious, but replication-deficient adenovirus vector. Using this Ad vector, they successfully infected and expressed the α1AT gene in epithelial cells of the rat respiratory tract *in vivo*. After intratracheal instillation of Ad-α1AT to test rats, α1AT messenger RNA was detected in the respiratory epithelium (Figure 1), and α1AT protein was produced and secreted by infected lung tissues. High levels of α1AT were also detected in the epithelial lining fluid, lasting at least 1 week. More recently, it was found that the expression of α1AT in test animals could be extended to several months.[5,8]

Using the same experimental system, Rosenfeld et al.[68] recently extended their studies by transferring the normal human cystic fibrosis transmembrane conductance regulatory (CFTR) gene to the airway epithelium. Expression of CFTR transcripts was observed for up to 6 weeks, and the protein was detected immunologically for about 2 weeks postinfection. These results suggest that optimized *in vivo* CFTR gene transfer may provide a therapy for cystic fibrosis. It was postulated that an aerosol spray mode might be more effective than the current perfusion method in dispersing the infecting vector over a broader surface area of the intratracheal tract, resulting in a clinically convenient treatment of certain lung diseases.

The technology of using adenovirus as a vector for human gene therapy has several advantages, as summarized by Rosenfeld et al.[18]: "(1) recombination is rare, (2) there are no known associations of human malignancies with adenoviral infections despite common human infec-

tion with various adenoviruses, (3) the adenovirus genome (double-stranded piece of DNA) can be manipulated to accommodate foreign genes of up to 7.5 kb in size of foreign genes, and (4) live adenovirus has been safely used as a human vaccine." Since this adenoviral infection method for lung epithelium employs a non- or minimally invasive procedure, it is a milestone toward gene therapy of various diseases, including common genetic disorders such as α1-antitrypsin deficiency and cystic fibrosis. Also, although respiratory tract epithelium has been established as the specific and natural target site for adenoviral infection, it may be useful to determine whether the epithelial lining of other organ types (e.g., digestive tract) can also be infected using high titer of virus preparations, thus providing additional means for gene transfer into internal organs.

Another approach using adenovirus for gene transfer is being evaluated by Cotton and Curiel.[69] In their experiment, polylysine was conjugated to disarmed Ad, then foreign DNA was bound to the polylysine, forming a complex for gene delivery. Both *LacZ* and *Lux* genes were successfully transferred in this manner to various human cell types *in vitro*. The transfer of genes is successful even if the AV genome is destroyed by UV irradiation, and Cotton and Curiel suggest that "it may be possible to get rid of the viral genome altogether," mainly because foreign genes are carried "outside" the virus particle. By using the AV coat protein (and particles) only as a vehicle for internalization of the transgene construct, this approach may overcome the safety concerns of using viral gene components in therapy and the size limitation on the DNA that can be delivered. It will be important to find out if the potential advantages projected by these researchers can be confirmed in future experiments.

Adeno-associated virus (AAV) has also been evaluated as a potential vector system for human gene therapy. Samulski et al.[70] demonstrated that efficient integration of the AAV viral genome into a single, specific site on human chromosome 19 (q13.4-ter) is a key step for AAV to establish latency when helper virus is absent. This finding indicates that the AAV may provide a site-specific integration strategy for human gene therapy.

D. *In Vivo* Gen Transfer t Brain by Herpes Simpl x Viru

Herpes simplex virus (HSV) is a neuronal tissue-specific virus. It can naturally persist in infected cells for a long latent period, during which only a limited number of its genes are expressed. This set of features suggests that recombinant HSV has the potential to serve as a vector for the transfer of foreign genes into the nervous system.

Recently, two research groups explored this possibility and obtained interesting results. Dobson et al.[19] used a recombinant HSV vector to inoculate mouse spinal ganglia via the sciatic nerve and hypoglossal nucleus via the tongue tissue. Deletion mutants of HSV vector were constructed to contain a *LacZ* gene driven by the mouse murine leukemia virus long terminal repeat. In whole-mount tissues, β-gal expression was shown in spinal ganglion in acute infections and in hypoglossal nucleus in latent infections.

Similar expression of β-gal in brain tissue via HSV-mediated gene transfer *in vivo* were obtained by Fink et al.[20] In this case, HSV vectors were delivered *in situ* by injection of virus preparations stereotactically into rat hippocampus and contralateral caudate. This group had previously shown that HSV mutants defective in the US3 gene exhibited an approximate 1000-fold reduction in their pathogenicity following intracranial inoculation.[71] They therefore inserted a β-gal gene driven by a viral late gene (glycoprotein C or gC) promoter so that it would disrupt the US3 gene product. With this recombinant HSV, designated US3 pgC-*LacZ*, the expression of β-gal would thus indicate the expression of foreign genes during viral replication. In another vector, d120 pLAT-*LacZ*, β-gal expression was driven by the viral latency associated transcript promoter (pLAT) and inserted into the gC locus of the deletion mutant. Expression of β-gal using this vector would thus indicate expression of foreign genes during viral latency.

This sophisticated experimental design worked as predicted. Using X-gal assays and immunocytochemistry straining of β-gal protein, they showed that β-gal expression in hippocam-

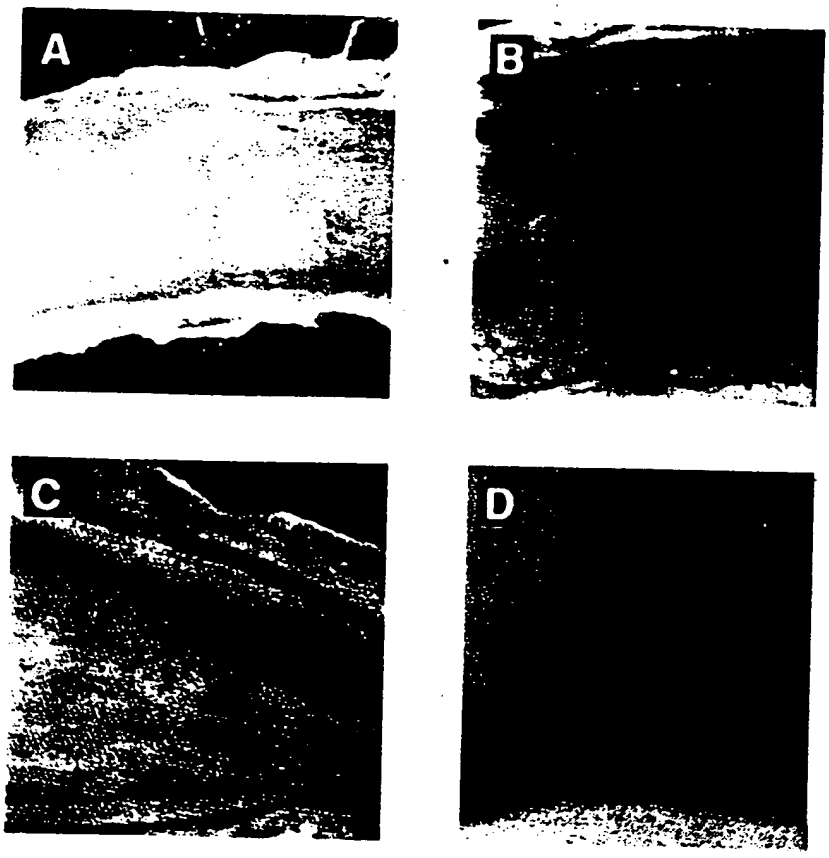

PLATE 1. Analysis of pig arterial wall infected with β-galactosidase-transducing retrovirus introduced *in vivo* by insertion of a catheter into the iliofemoral artery. β-Galactosidase activty was documented by histochemical staining in a segment of (A) normal control artery that was sham-infected or segments infected directly with the replication-defective β-galactosidase retroviral vector and analyzed after (B) 8 weeks or (C) 21 weeks, and (D) segments transduced by liposome transduction after 4 d. (Reprinted by permission of the publisher from Nabel et al., *Science*, American Association for Advancment of Science, Washington, D.C., 1990.)

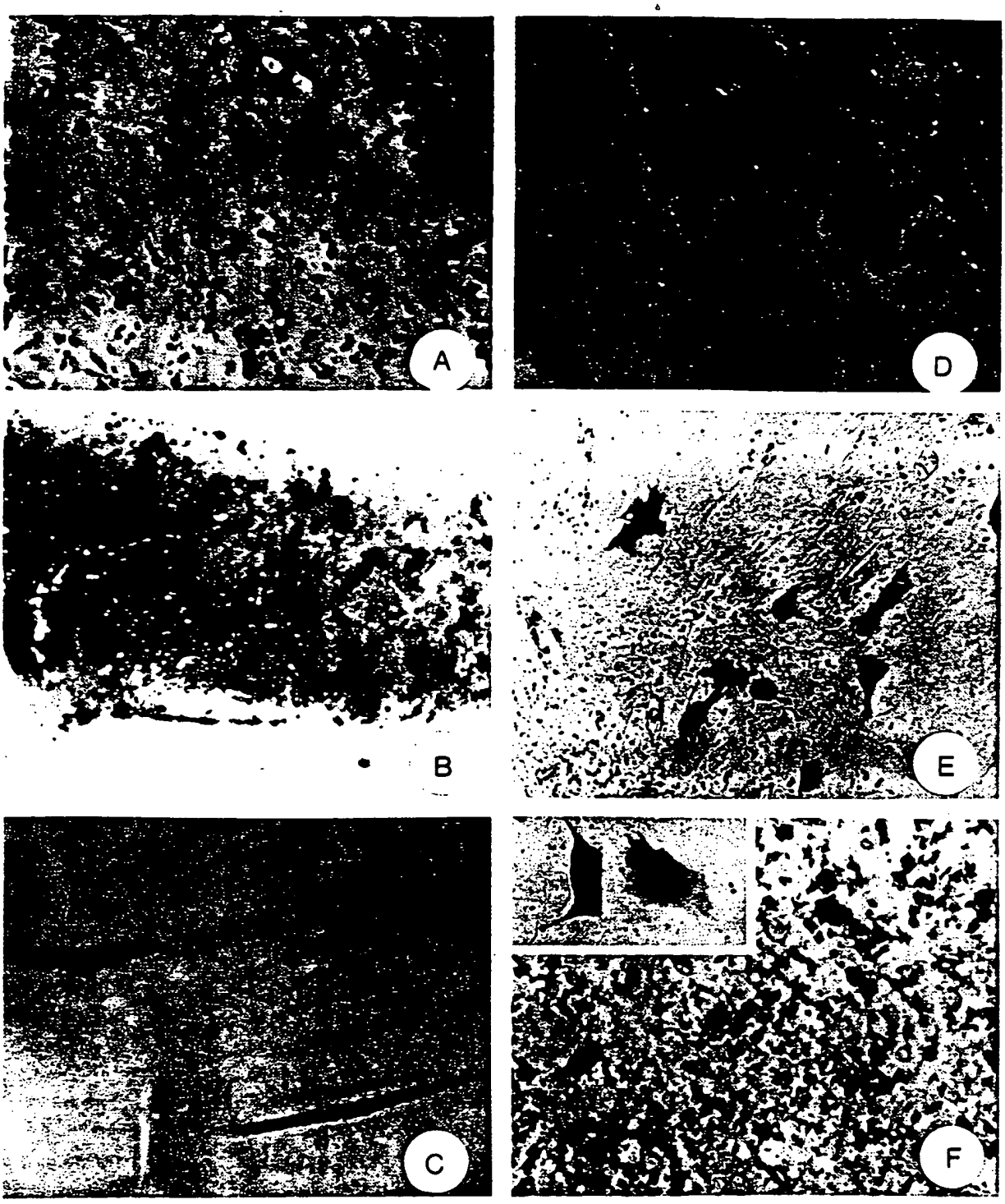

PLATE 2. Expression of CMV-β-Gal or MMTV-β-Gal activities observed at the cellular level for various bombarded tissues. Cells that are stained blue indicate β-galactosidase activity. (A) Mouse skin tissues bombarded *in vivo* with CMV-β-Gal DNA. (B) Rat liver tissues bombarded *in vivo* with CMV-β-Gal DNA. (C) Mouse muscle tissues bombarded *in vivo* with CMV-β-Gal DNA. (D) Rat mammary gland organoids bombarded *in vitro* with MMTV-β-Gal DNA. (E) Primary cultures of human mammary epithelial cells bombarded with MMTV-β-Gal DNA. (F) Human mammary carcinoma cell line (MCF-7) cells bombarded with MMTV-β-Gal DNA: Inset at high magnification. (Adapted from Yang et al., *Proc. Natl. Acad. Sci. U.S.A.*, National Academy of Sciences, Washington, D.C., 1990.)

pus was readily detected with the US3 pgC-*LacZ* vector at 2 to 3 d after HSV inoculation, with activities disappearing by day 7. Injection of the d120 pLAT-*LacZ* vector into the hippocampus did not result in focal expression of β-gal in injected tissues. Instead, isolated and scattered β-gal expressing cells were observed throughout the brain between 1 week and 3 months postinjection. Molecular analysis showed that viral genomic DNA and *LacZ* mRNA sequences were present in the brain 12 d postinjection. These results suggest that the LAT promoter was controlling β-gal expression during viral latency.[20]

The results of Fink et al.[20] and Dobson et al.[19] complement each other, supporting future experimentation of the HSV vector for brain gene therapy. Since these HSV-mediated gene transfer systems are just beginning to be explored, we may optimistically expect that future studies will generate information that will be useful not only to gene transfer technology, but also to brain research.

One major concern about human gene therapy is its safety. Although Friedmann and coworkers[72] showed that high levels of cytopathogenicity were detected when certain recombinant HSV constructs were used to transduce nerve or other cell types under *in vitro* conditions, Fink's and Dobson's *in vivo* experiments indicated that neuropathogenicity in treated brain tissues were minimal. In animals of up to 10 months postinoculation, Fink et al.[20] observed only limited disruption of the normal architecture of neuronal tissues in hippocampus. The difference in vector design and constructions used between *in vitro* and *in vivo* experiments may be a cause of the high level of cytotoxicity observed *in vitro*. Alternatively, the physiological and cellular environments of *in vivo* vs. *in vitro* brain tissues may be different enough that certain recombinant HSV vectors are intrinsically less pathogenic *in vivo* than under *in vitro* conditions. This remains to be verified in future experiments.

III. PHYSICAL MEANS FOR DIRECT GENE TRANSFER *IN VIVO*

A. Direct Gene Transfer into Muscle

Occasionally, presumed negative control samples included in experimental sets yield unexpected results. This was the case that led to the discovery of an important *in vivo* gene transfer method that is useful for muscle tissues. Wolff et al.[11] attempted gene transfer using a precipitated lipofectin-DNA complex directly injected into muscles of live experimental mice. But results obtained from injecting DNA alone were repeatedly found to have higher gene expression than those from injecting lipofectin-DNA complex. Eventually, they determined that simply by injecting a plasmid DNA solution into mouse quadriceps, high level and sustained expression of marker genes could be obtained in injected muscle tissues.[11] Gene expression level was found to be dependent upon the dosage of DNA injected. One week after DNA delivery up to 300 pg of Lux activity per target site were detected. Wolff's group initially reported that these levels were maintained for 12 weeks, but more recently they found that gene expression in some animals continued for more than 18 months, with marker gene activity in certain test animals increasing steadily throughout the experimental period (J. Wolff, personal communication). Histological studies show that clusters of striated muscle tube cells express high levels of β-gal activity.

The most intriguing finding of these muscle experiments was that the long lasting fate of gene expression was apparently not the result of foreign gene integration into the muscle cell genome. Instead, Wolff et al.[11] demonstrated by Southern blot analysis (Figure 2) that months following DNA delivery, the introduced marker genes still persisted as nonintegrated, circular plasmids that had not replicated. Therefore it was shown for the first time that certain terminally differentiated, nonproliferative tissues (e.g., muscle) could effectively maintain introduced functional genes *in vivo* in plasmid form for a prolonged period of time. Although they were not integrated into the target cell genome, these long-lasting plasmid DNA in muscle cells continued to express the functional genes they carried.

For certain gene transfer or gene therapy purposes, nonintegrative foreign gene expression may be preferable since, for example, integration-associated genomic rearrangement may lead to undesirable mutations in host genes. Also, simple injection of DNA into muscle is apparently easy to perform repeatedly. Therefore, long-term, but not necessarily permanent, transfer of therapeutic

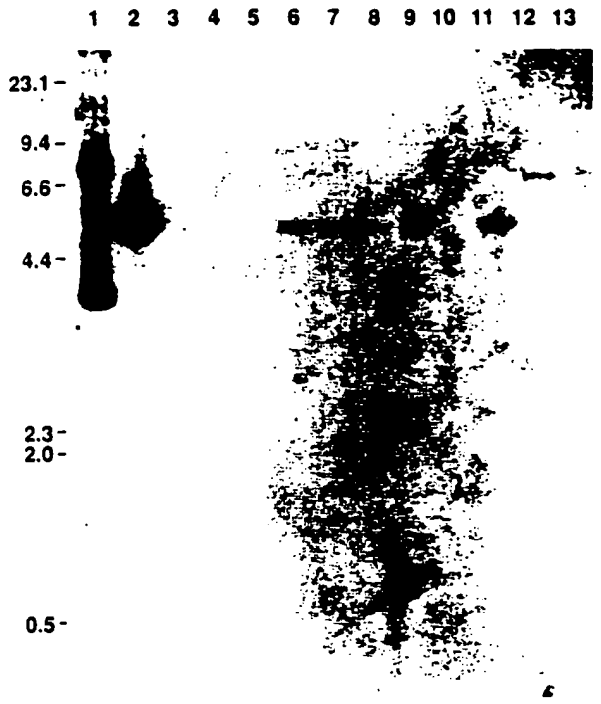

FIGURE 2. Southern Blot analysis of DNA from quadricep muscle injected 30 d previously with plasmid DNA containing RSV-Lux construct (pRSVL). Hybridization is with multiprimed ^{32}P-labeled luciferase probe from pRSVL. Lane 1, 0.05 ng of undigested pRSVL plasmid; lane 2, 0.05 ng of Bam HI-digested pRSVL; lane 3, empty; lane 4, Bam HI digest of Hirt supernatant from control muscle; lane 5, Bam HI digest of cellular DNA from control, uninjected muscle; lanes 6 and 7, Bam HI digest of Hirt supernatant from two different pools of pRSVL-injected muscles; lanes 8 and 9, Bam HI digest of cellular DNA from two different pools of pRSVL-injected muscle; lane 10, cellular DNA (as in lane 9) digested with Bam HI and Dpn I; lane 11, cellular DNA (as in lane 9) digested with Bam HI and Mbo I; lane 12, cellular DNA digested with Bgl II; and lane 13, Hirt supernatant digested with Bgl II. Size markers (/Hind III) are shown on the left in kilobases. (Reprinted by permission of the publisher from Wolff et al., *Science*, American Association for Advancement of Science, Washington, D.C., 1990.)

genes into muscle may provide a potential treatment for certain genetic deficiencies of muscle functions (e.g., Duchenne's Muscular Dystrophy). Using this method, Acsadi et al.[73] reported that low level expression of a transgenic human dystrophin gene was detectable in DNA-injected mouse muscle.

The DNA injection method for mouse quadricep muscles has been reported recently to be applicable to heart muscle tissues, but seems to be minimally effective for smooth muscle or other organ types.[12] More recently, Jiao et al.[74] showed that the direct DNA injection method seems to work best in mice, less well in rats and cats, and least in primates, suggesting potential limitations for human use. The biochemical and cellular mechanism(s) by which plasmid DNA molecules are being taken up by muscle cells *in vivo* is not clear. It is possible that transverse (T) tubules present in rough muscle fiber cells may provide the passage for DNA uptake. If so, it would explain why smooth muscle types, which do not possess T-tubules, were found to be minimally susceptible to this DNA injection method. Tracing of fluorescence labeled DNA or *in situ* hybridization at the cellular level may reveal such information in future experiments.

Another example of direct injection of DNA into tissues *in vivo* and the resulting functional expression of a foreign gene in target tissue was reported many years ago by Fung et al.[30] In this case, Rous sarcoma virus subgenomic DNA or cloned v-*src* DNA was directly injected into the wing-web of newly hatched chickens. Sarcoma tumors were generated in test birds at 3 to 4 weeks after DNA injection, with an incidence rate of $\geq$60%. Southern blot analysis showed that these tumors had acquired v-*src* sequences in the genome and expressed high levels of src messages. Both subgenomic and cloned v-*src* genes gave similar results. This study was the first to show that direct injection of cloned DNA into animal tissues *in vivo* can result in uptake, integration, and stable expression of introduced genes. Because the v-*src* gene codes for a tyrosine-specific protein kinase and is capable of transforming chicken or mouse fibroblasts, they also demonstrated that v-*src* gene alone is oncogenic *in vivo* in the avian system. Recently, England et al.[31] showed that direct injection of v-*src* DNA fragments intraperitoneally into chicken induced mesothelioma, suggesting that, at least in avian hosts, mesothelium of the peritoneal lining tissue is effective for *in vivo* uptake of naked DNA molecules.

B. Particl Bombardment-Mediated Gene Transfer

Particle-mediated gene transfer methods were first applied to the transformation of plant tissues.[75,76] With a particle bombardment device, a motive force is generated to accelerate the DNA-coated gold particles to high velocity, enabling efficient penetration of target organs, tissues, or single cells. It was thought that because this method employs a physical means for direct delivery of DNA into plant cells, either into the cytosol or into the nucleus, it could circumvent the need for interactions with cell wall for cell membrane receptors, which often exhibit cell-type specificity for vector- or chemical-mediated gene transfer methods. Indeed, later experimentation confirmed this theory.[76] One of the most important contributions of the gene-gun technology was demonstrated by Christou et al.[76] They determined that the apical meristem (the growing point tissues) of plant seedlings can be used effectively as target tissues for direct gene transfer via the particle bombardment method. As a result, chimeric plant tissues, organ segments, whole organs, and clonal plants were transformed using this gene transfer method.[76] This technology has proven to be useful for a wide variety of plant tissues and species, and has been used for the production of transgenic soybean, cotton, bean, rice, and corn (P. Christou, D. McCabe, D. Russell, and G. Brar, personal communications).

Figure 3 shows the design of an electric discharge-mediated particle acceleration device that was used initially for plant gene transfer, as reported by Christou et al.[76] It was later modified and adapted for gene transfer to various mammalian somatic tissues as described by Yang et al.[21] An important feature of the design for this device is that the particle acceleration force can be fine-tune regulated as a function of the discharge voltage. Therefore, depending on specific needs for *in vivo* target tissues or for *in vitro* cultural conditions, the penetration of gold particles can be carefully adjusted to optimize DNA delivery.

With this particle bombardment method, it was demonstrated that gene transfer to various mammalian somatic tissues can be effectively achieved in *in vivo*, *ex vivo*, and *in vitro* systems.[21-23] In the case of *in situ* bombardment of various organs of live animals, it was demon-

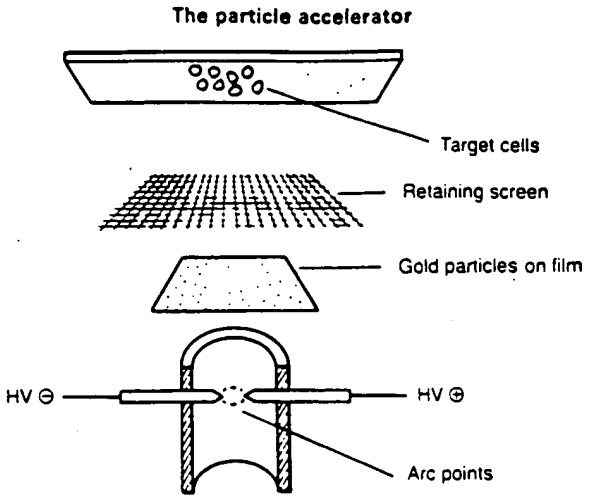

FIGURE 3. The motive force is generated in a spark discharge chamber containing two electrodes. A 10 μl water droplet is placed in between the electrodes, and a high-voltage capacitor is discharged through the water droplet that vaporizes instantly, creating a shock wave. We have found that a polyvinyl chloride pipe with an internal diameter of 13 mm is adequate for use at the spark discharge chamber. The electrodes are located opposite each other, project into the interior of the chamber approximately 5 mm below the top and are protected at the tips with an arc-resistant alloy. The gap between the two electrodes can be adjusted by appropriately threading them into or out of the spark chamber. A spacer ring is placed above the spark chamber that, in a fixed apparatus for transformations of a single crop species, can be a vertical extension of the spark discharge chamber. However, a removable spacer ring allows the distance from the spark discharge to the carrier sheet to be varied so that the force of the shock wave can be adjusted. The motive force can also be adjusted by varying the voltage of the discharge. The carrier sheet on which the DNA-coated gold particles are precipitated is placed on top of the spacer; the function of this sheet is to transfer the force of the shock wave from the spark discharge into acceleration of the carrier particles. Located above the carrier sheet is a 100-mesh stainless steel screen that retains the sheet so that it does not proceed to the target tissue. The target tissue can be placed on a water-agar plate in such a way that when the plate is inverted over the retaining screen the tissue is in the direct path of the gold particles. The whole assembly is under a partial vacuum in order to minimize aerodynamic drag. (Reprinted by permission of the publisher from Christou et al., *Trends Biotechnol.*, Elsevier, Cambridge, 1990.)

strated that particle bombardment technology can efficiently deliver foreign genes into skin and liver and muscle tissues of rat or mouse *in vivo*. Plate 2 (A to C) shows that expression of the *LacZ* gene in animals can be readily detected in targeted organs at 1 to 2 d following gene transfer. More recently, this result was extended to many other organs, including pancreas, kidney, spleen, and blood vessels (Yang et al., manuscript in preparation). For certain organs, expression of reporter genes, including *Lux* and *LacZ*, has lasted for 2 to 12 months following gene transfer, with maintenance of more than 40% of the maximal activity (detected at 1 d after bombardment). The particle-mediated gene transfer method can therefore confer long-term gene expression in certain target tissues. We have not determined whether the long-term *in vivo* gene expression observed for certain organs was a result of the integration of marker genes into the host cell nuclear genome or of the stability of plasmid DNA *in vivo*. Both seem possible, as was discussed in the previous section.

Availability of such an efficient system for *in vivo* gene transfer and transient expression, which is applicable to a variety of mammalian organs, makes it possible to assay relative strength of promoters among different organs without having to go through time-consuming germ line transgenic animal systems. Also, since the generation of transgenic animals, other than mice, is still technically very difficult, the particle gene transfer method offers the opportunity to study relative promoter strength in non-mouse, experimental animals. Toward this goal, *in vivo* promoter activity experiments, using *Lux* as a reporter gene, were performed in rats to evaluate expression of various viral and cellular promoters in different tissues (Yang et al., submitted). Two cellular promoters, phosphoglycerate kinase (PGK) and phosphoenolpyruvate carboxykinase (PEPCK) were found to exhibit interesting patterns for tissue-type expression. Results showed, for example, that *in vivo* transient expression of PGK and PEPCK promoters was 10- to 20-fold more efficient in skin than in liver or pancreatic tissues. Based on these results, it is suggested that the particle gene transfer method may be employed as a useful *in vivo* assay system for evaluating tissue specificity and relative strengths of promoters. Particle-bombardment gene transfer performed *in situ* on live animals may also offer a fast and convenient assay for the construction and the effect of therapeutic genes in target tissues of test animals.

Using particle bombardment for gene transfer, Tang et al.[77] have demonstrated that the technology also provides a method for genetic immunization. In their study, mouse ear tissue was bombarded *in vivo* to transfer plasmids containing hGH or α1AT genes. After 3 to 8 weeks, antibodies against hGH or α1AT were detected at high titer in serum of the genetically inoculated mice. This technique thus offers a simple method for eliciting a humoral immune response and it may also provide a means for genetic vaccination against pathogenic infections.

Particle bombardment technology can also be applied to *ex vivo* solid tissue explants, organ slices, or organoids.[21,23] An example is shown in Plate 2D, where freshly isolated rat mammary ductal segments (organoids) were bombarded, and expression of β-gal was readily detected at the cellular level 1 d later. Such bombarded *ex vivo* tissue materials can be monitored *in vitro* for specific experimental purposes (e.g., transgene behavior in primary cultures), or transplanted back into the body for gene therapy or other clinical purposes.

In addition to *ex vivo* and *in vivo* solid tissues, the particle bombardment method has also proven useful for *in vitro* gene transfer in cell cultures.[21,23,78,79] Various mammalian primary cultures, including those derived from the mammary gland (Plate 2E), liver and brain, were effectively bombarded to transfer a variety of marker genes, whose expressions were readily detected (Reference 20 and Yang et al., unpublished data). It is well known that many types of mammalian primary culture systems are difficult to maintain and to establish into low passage cultures. This is due to several technical difficulties: (1) surgically excised and enzymatically dissociated tissues are often obtained as cell aggregates or tissue clumps, (2) cells are difficult to remove from or reattach to culture substratum, and hence difficult to transfer into subcultures, and (3) cells are often very sensitive to changes of culture medium, treatment with chemicals, or other tissue culture manipulations. As a result, unlike established cell

culture lines, primary cell cultures can often be quite inefficient or unreliable to transduce using various gene transfer methods. Since the bombardment method involves very simple culture procedures (i.e., withdraw medium from culture, bombard cells, then add the conditioned medium back to culture — the whole procedure often takes less than 30 s to complete), it was found to be quite effective for some primary cultures.[21,23] Thus, the particle bombardment technology may prove to be a useful alternative for gene transfer to a variety of primary cell cultures. This may also have implications for some gene therapy procedures, in which cell or tissue samples from patients need to be grown out in primary cultures for gene transfer before being transplanted into patients.

Gene transfer and transient gene expression via particle bombardment has also been demonstrated in eight human cell culture lines, including cells of epithelial (Plate 2F), endothelial fibroblast, and lymphocyte origin.[21,78] Using CHO and MDF-7 cell cultures as models, stable gene transfer was obtained at frequencies of 1.7×10^{-3} and 6×10^{-4}, respectively.[21]

For potential usage in industrial or medical biosystems, the particle bombardment method may offer another practical advantage over other means of gene transfer, which is the capacity to deliver high dosage of DNA into target cells. We have determined that transient gene expression level increases at a linear rate proportional to the amount of DNA loaded onto the gold particles.[21] At a level of 1 μg of DNA per mg of gold particles, this DNA loading rate corresponds to 10,000 copies of a 5-kb DNA molecule per gold particle (1 to 3 μm). Because DNA molecules are precoated onto particles in an ethanol precipitated form, such DNA molecules on gold particles are in a dry, solid form, in contrast to being in solution. DNA-coated gold particles can deliver the predetermined and high amount of DNA into targeted cells in an effective and precise manner, for example, an average of 10,000 copies per bead for each target cell. Hence, the particle bombardment method is a practical way to achieve high-dosage DNA delivery and the resulting high expression levels.

In summary, the particle-mediated gene transfer method for delivery of foreign DNA to mammalian cells is possible for a wide range of cell types and cell environments. Since the direct physical delivery of DNA into the nucleus or cytoplasm obviously bypasses specific barriers due to membrane receptors, this method may serve as a general method for gene transfer into various mammalian somatic cells. It may provide a useful alternative to the retrovirus-based vector systems. Since it can be used *in vivo* for liver, skin, muscle, and several other organ types, many types of solid tissues or organs can now be considered target tissues for direct gene transfer. This method can also be applied *ex vivo* to various tissues, including surgically excised or biochemically dissociated organ segments or tissue clumps, as well as their derivative primary cultures. The particle bombardment procedure involves minimal manipulation of target organs, tissues, or cells and is versatile, efficient, and flexible. With further optimization of involved techniques, this technology may prove applicable to various aspects of somatic cell gene therapy.

C. Electroporation for Gene Transfer into Skin

Electroporation has been used extensively for gene transfer into a variety of cell cultures *in vitro*. Titomirov et al.[29] showed recently that it can also be applied to mouse skin tissues *in vivo*. Prior to electroporation, plasmid DNA containing the NPT-II marker gene was injected subcutaneously, thus was exposed to the dermal layer of skin tissue. Using a device that clamped electrodes onto taut skin tissue, they applied a field strength of 400 to 600 V/cm across the skin epidermal and dermal tissues, with a pulse time of about 150 μs. Twenty-four hours after electroporation the skin tissues were excised, and primary cultures of skin fibroblast cells were established. G418 selection confirmed that a significant population ($\sim 10^{-4}$) of electroporated skin cells was thus stably transduced by this method combining *in vivo* gene transfer and *in vitro* cultivation and selection. These results demonstrated that, with the appropriate system for tissue assembling, some tissues can be made susceptible to electroporation-mediated gene transfer *in vivo*. However, until significant levels of gene expres-

sion can be directly detected in live animals, it will be difficult to evaluate whether electroporation is practical for *in vivo* gene transfer.

IV. CARRIER-MEDIATED GENE TRANSFER *IN VIVO*

If therapeutic genes can be conveniently introduced into body fluids or the bloodstream, and then be directed *in vivo* to target a specific organ as the final site for functional gene expression, this route of gene transfer may have many applications for somatic gene therapy. This was the approach pursued by several laboratories, including those of Nicolau,[26] Huang,[27] and Wu.[24,25] Both liposomes and polycations (e.g., polylysine) were evaluated as carriers for *in vivo* DNA delivery.

A. Targeting Gene Transfer with Immunoliposomes

Pilot studies using liposome entrapped DNA for *in vivo* gene transfer were reported by Nicolau and co-workers.[80] They demonstrated gene expression in rat livers that had received an intravenous injection of the preproinsulin I gene encapsulated in conventional, pH-insensitive liposomes. Later, Soriano et al.[26] showed that liposomes bearing galactosyl groups were targeted with detectable specificity to hepatocytes *in vivo*. These results prompted other investigators to follow their examples and pursue liposome- or polylysine-mediated, target cell-specific delivery of foreign genes *in vivo*.[24,25,28]

Liposomes release their entrapped contents into the cytoplasm of target cells after they fuse with endosomal membranes.[81] To increase specific binding of liposomes to target cells *in vivo*, Wang and Huang[27] incorporated acylated monoclonal antibodies into the lipid bilayer of pH-sensitive liposomes. Their results indicated that this approach can significantly improve specificity for targeting gene expression *in vivo*. They used a chloramphenicol acetyl transferase (CAT) gene construct driven by a cAMP-regulated promoter, entrapped in H2-K^k antibody-coated liposomes. In this model system, they used RDM-4 lymphoma cells, which express the mouse major histocompatibility antigen H2-K^k, as the target cells for *in vivo* gene transfer. These cells were grown as ascites tumors in immunodeficient BALB/c mice. Then immunoliposome-entrapped DNA or free DNA were injected intraperitoneally into nude mice. At 24 h after DNA injection, about 20% of the immunoliposomes were found to be taken up by the target RDM-4 cells. Uptake was much less when control liposomes (i.e., without antibody) were used. Spleen and stomach tissues and adherent ascites cells were found to be the major sources for nonspecific uptake of liposomes.

Significant CAT activity was detected in RDM-4 cells from mice treated with pH-sensitive immunoliposomes, and the expression was found to be responsive to treatment with cAMP. CAT expression was also detected in the liver and spleen, but at much lower levels and it did not respond to cAMP treatment. It was not clear what mechanisms contributed the cAMP-independent CAT expression in these tissues. They also showed that pH-sensitive immunoliposomes were severalfold more efficient than pH-insensitive liposomes for delivering the CAT gene into the RDM-4 cells.

This study showed that pH-sensitive immunoliposomes may have applications in targeting specific tissues for *in vivo* delivery and expression of foreign genes via intraperitoneal DNA injection. However, if the uptake of liposomes by spleen and stomach tissues cannot be avoided, it may compromise this strategy for targeting gene therapy for certain diseases.

Although varying degrees of initial success with *in vivo* gene transfer via liposomes were obtained by Huang's and Nicolau's groups,[26,27] future experiments to determine the time course and relative levels of transgene expression in these liposome systems are needed.

B. Targeting Gene Transfer with Asialoglycoprotein/Polylysine

Wu et al.[24] more recently showed that marker DNA complexed to another carrier, the asialoglycoprotein/polylysine conjugate system, can target hepatocytes for *in vivo* gene transfer. Their

work was based on these previously observed findings: (1) normal hepatocytes contain unique cell surface receptors that recognize and internalize galactose-terminal (asialo) glycoproteins,[82] and (2) poly-L-lysine can bind DNA without damaging it to form strong, soluble complexes.[83] Thus, they designed a DNA delivery system targeting liver tissue by isolating orosomucoid from human serum, desialylating it to form asialoorosomucoid (ASOR), and then conjugating ASOR to polylysine (Mt = 3800). They then added this conjugate to form a complex with the plasmid DNA, which contained a CAT gene under the control of specific promoters (SV40 or albumin).

These DNA-carrier complexes were delivered into the mouse bloodstream via tail vein injection and transient CAT enzyme activity was detected in liver tissue extracts 24 h later. The maximum level was 10 units per g liver at 24 h, with activity falling to nondetectable levels by 96 h. In contrast, when partial hepatectomy was performed on the test animals, persistent levels of hepatic CAT activity were detected in the liver up to 11 weeks after the injection (Figure 4). These results[24,25] are encouraging because they show that DNA delivered as a protein complex can be efficiently targeted to liver tissues for sustained expression *in vivo*. However, because partial hepatectomy is a rather invasive surgical procedure, it may compromise the original goal of noninvasive gene therapy.

In these experiments, Wu et al.[24,25] first demonstrated the profound effect of partial hepatectomy on foreign gene expression patterns. Without partial hepatectomy, CAT activity was low, declined rapidly, and ultimately disappeared in 4d, but with partial hepatectomy, CAT activity was sustained for 11 weeks. They also reported that in some test samples the input DNA had been integrated into the host cell genome.[24] Since it was not clear what percentage of these cells were present in the regenerated liver, it cannot be determined whether integrative gene transfer alone could account for the sustained expression level. Therefore, in future experiments, it will be important to determine the extent of gene expression in liver tissues at the cellular level, especially the level of sustained gene expression. This information will also be necessary for evaluating practical applications of this procedure for clinical gene therapy of liver deficiency.

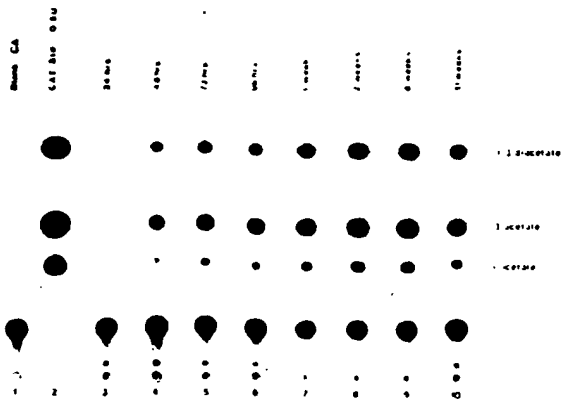

FIGURE 4. The effect of partial hepatectomy on targeted CAT gene expression. Rats were injected with AsOR-poly-L-lysine-DNA complex. A 66% partial hepatectomy was performed 30 min later, and at various time points livers were assayed for CAT activity. (Reprinted by permission of the publisher from Wu et al., *J. Biol. Chem.*, American Society for Biochemistry and Molecular Biology, Bethesda, MD, 1989.)

More recently, the same group (Wilson et al.[84]) utilized ear vein injection of the targetable DNA protein complexes to deliver a human LDLR gene into LDLR-deficient rabbits. In this study, partial hepatectomy was not performed, so the gene delivery process was not invasive. They demonstrated that high quantities of DNA can be efficiently delivered to the liver, but more than 99.9% of plasmid DNA was apparently degraded in 48 h following uptake by the liver. However, the initial high dosage of delivered DNA produced sufficient LDLR to significantly decrease the serum cholesterol level for at least 6 d following gene transfer. This noninvasive method of *in vivo* gene transfer may therefore be applicable to brief, repeated gene therapy treatments.

C. Targeting the Nucleus for Gene Transfer

Kaneda et al.[28] developed a sophisticated protocol that permits entrapment of DNA coupled with carrier nuclear proteins into specifically engineered vesicle complexes. When marker genes

were loaded into these vesicles and injected into the portal veins of adult rats, plasmid DNA was found to be efficiently carried into liver cell nuclei. Reporter genes coupled with nuclear proteins were expressed at least five times more efficiently in rat livers than the genes carried by nonnuclear proteins. Nuclear proteins that were shown to confer this effect included high-mobility group-1 protein (HMG-1, a nonhistone chromosomal protein) and nucleoplasmin (DNA binding protein extracted from *Xenopus laevis* oocytes). Bovine serum albumin was employed as the nonnuclear protein control. Marker gene expression in hepatocytes was detected at the cellular level 4 d following injection of vesicle complexes into liver.

These results suggest that nuclear proteins co-introduced into liver tissues *in vivo* can facilitate the migration of foreign DNA into the nucleus, resulting in increased levels of transient, nonintegrative gene expression at the cellular level. In the future this strategy of targeting the nucleus for gene transfer at the subcellular level may be adapted to optimize other methods of *in vivo* gene transfer.

V. RECENT PROGRESS IN *IN VITRO* GENE TRANSFER TECHNIQUES

Electroporation for gene transfer was first developed for the transduction of lymphocyte cell cultures.[85] Since then, virtually all established electroporation methods were designed for usage in tissue culture samples prepared as cell suspensions. Recently, Zheng and Chang[86] demonstrated that by using a pulsed radiofrequency electric field, high-efficiency gene transduction can be obtained with cultured mammalian cells in their attached state (*in situ*). At optimal electric field strength (~1.2 KV/cm), over 70% of the M6 test cells took up and expressed the β-gal gene, with a cell survival rate of about 80%. In contrast, less than 20% of M6 cells were transiently transduced when these cells were electroporated in suspension. Time course data suggested that the difference in gene transfer efficiency was due to the effect of the cell detachment treatment. Therefore, this new method for electroporation of cultured cells *in vitro* appears to be more efficient and convenient for some attached cells.

Chang and Reese[87] demonstrated that electroporation does indeed create pores in the cytoplasmic membranes of treated cells. Rapid-freezing electron microscopy revealed that, at 40 ms, pore-like openings were observed in cell membranes with diameters of 20 to 120 μm. These membrane pores were drastically reduced in size after 5 s, and by 10 s they had disappeared and were replaced by pit-like indentations in the membrane. Their findings provided good physical evidence that a brief (~5 to 10 s) opening of pores is most likely the mechanism responsible for gene transfer by electroporation.

Sasaki et al.[88] recently described a simple technique to introduce DNA into cells through cracks and/or pores made in cell membranes caused by intracellular ice crystal formation upon brief freezing in liquid nitrogen. Techniques and conditions were optimized for these brief freezing treatments. They showed the procedure resulted in only moderate cell killing and was effective in generating G418-resistant cell colonies transduced with a NPT-II gene as the selectable marker.

Since the methods of attached cell electroporation, brief freezing, and particle bombardment all employ a physical means for DNA delivery into cultured cells, they may circumvent cell or membrane-receptor specificity for DNA uptake. These methods may prove to be useful for gene transfer to certain cell types which are difficult to transduce by viral or liposome-mediated gene transfer methods.

Wagner et al.[89] reported recently that transferrin covalently linked to polycations (protamine or polylysine) can serve as a useful carrier for DNA uptake into erythroblasts in culture. Electrophoretically stable complexes were formed between the transferrin/polycation molecules and various DNA or RNA molecules, independent of their molecular size (up to 21 kb of DNA). Using a luciferase gene as a marker, they showed that high levels of gene expression were detected in erythroblasts, demonstrating that transferrin receptor-mediated endocytosis is efficacious for gene transfer.

VI. CONCLUDING REMARKS AND FUTURE PROSPECTS

It is obvious that within the past couple of years extensive progress has been made on *in vivo* gene transfer and expression technology. Important findings were obtained not in just one or two specific experimental systems, but in half a dozen different areas. Very diversified tools and means were established. Through these developments we are seeing the heretofore scientific and technological barriers fall away. Many scientists believe these developing technologies will have a strong and long-lasting impact on our future thinking and on the direction of gene therapy. Researchers and clinicians involved in gene transfer are being motivated by their own new findings to think further about practical and clinical applications of specific gene transfer methods. The boundary between basic science and applied technology has become blurred so that often new developments in technology have become a driving force for new directions in basic research.

Due to the fast pace of technological development, experimenters must be extremely careful in their interpretation of observations. Whereas appropriate negative controls may lead to very useful new findings (e.g., gene transfer into muscles *in vivo*), lack of appropriate control or parallel experiments can often mask the true meaning of certain observations. This may be especially true for whole animal experiments, because whole body physiology as well as the target organ anatomy can vary among different organisms, and under certain experimental conditions can vary considerably among individual animals. One cautious observation on the recent development of *in vivo* gene transfer is that, while many novel techniques are being reported, some of them are very difficult for other laboratories to adapt. Although there is understandably a high constituency of "art" in any pilot development in technology, systematic and parallel experimentation will still serve as the best approach for resolving many of these technical difficulties.

There are two key characteristics that I perceive as critical for future assessment of specific applications of gene transfer methods. First, we need to use a normalized, relatable term (i.e., specific activity) to describe gene expression levels. At present, expression levels of CAT, *LacZ*, *Lux* and other reporter genes are often being reported on a nonquantitative, arbitrary scale, which prevent comparisons between different gene transfer systems. Levels of gene expression in specific activity, expressed as the percent of transduced cells in treated cell or tissue samples, or as nanogram of transgenic protein produced per milligram of protein in tissue extract or per million test cells, can be effectively employed by other investigators to relate results to each other, avoiding ambiguity. Furthermore, these measurements, when collectively accumulated among different researchers, will serve as baseline criteria for future studies on physiological effect of various therapeutic genes. Secondly, it is also important to establish the time course of gene expression, not only for the long term, but also for the short term. Some gene transfer methodologies may provide very long-term expression, but expression levels may be low. However, such cumulative effects may be quite suitable for certain approaches of gene therapy. Conversely, an extremely high level or a burst of gene expression restricted to a short period of time may be more suitable in clinical application to some other diseases. It would thus be too early to disregard any of the currently available gene transfer methods in terms of their potential utility for gene therapy. Combinations of different methods and regimes for gene transfer could very well be profitable in certain diseases. With these considerations in mind, researchers have the opportunity and responsibility to identify the applications of various developing gene transfer methods, to optimize the techniques for specific goals, and to combine different gene transfer methods for better utility.

Although it has been minimally addressed in this review, it is apparent that there are many applications of *in vivo* gene transfer technology to various areas of basic research. It is recognized that many physiological and biochemical effects observed under *in vitro* conditions cannot be applied or demonstrated under *in vivo* conditions, and the converse may also be true. Therefore, when technically and economically possible, metabolic and physiological effects eventually have to be tested under *in vivo* conditions. *In*

vitro or *ex vivo* model systems do have their applications, but they are limited. This applies to studies on gene expression and regulation at the organ or tissue level, especially for genes that are known or expected to play a key role in physiology or metabolism. With recent advances of *in vivo* gene transfer methods, it is now possible to approach some of these experiments without having to utilize germline transgenic animal systems (which can often be time-consuming, costly, and limited to few experimental systems). Influence of foreign gene expression on growth, differentiation, and neoplastic transformation of specific tissues or organs may now be studied *in vivo* by using "transgenic tissue."

Any integrative gene transfer, other than site-specific homologous recombination, may generate mutagenic or even carcinogenic effects. The probability of this risk can be assessed with a large body of experimental evidence. The strongest argument that this probability is low is that foreign gene sequences are integrated into non-homologous sites on chromosomes in a random fashion. Also, drastic mutations of functionally important genes (including proto-oncogenes) are known to often cause cell death, eliminating these cells from the *in vivo* transduced cell population.

To minimize or eliminate these mutagenic or carcinogenic effects, researchers can adapt the homologous recombination strategy for gene transfer.[90,91] This area of research has made extensive progress recently, especially with the use of double selection methods to increase selection efficiency of correctly transduced cells.[91,92] The primary challenge in using site-specific or homologous recombination is that *in vivo* gene transfer methods must be optimized so that they will produce sufficient levels of gene expression in spite of reduced DNA recombination efficiency. Secondly, *in vivo* selection systems must be developed to eliminate random gene insertions. Increased gene delivery efficiency combined with *in vivo* selection systems would make homologous recombination practical for *in vivo* gene transfer.

In vivo technology to transfer functional genes into experimental animals is progressing at a very fast pace. Scientists and clinicians are now responsible for identifying specific clinical applications and for optimizing various gene transfer techniques to effect gene therapy. Conceived some 20 years ago by W. F. Anderson, T. Friedmann, and other pilot researchers, gene therapy is now accepted as a feasible approach for the treatment of certain genetic diseases and cancer.[93] Many researchers in this field believe that the future of gene therapy is very encouraging, though the course will certainly not be straightforward. There are still many technical problems that need to be addressed at this early stage of technology development.[94] However, with recent advances in cellular and molecular biology, tissue engineering, and gene transfer technology, many scientists and clinicians are optimistic that future breakthroughs are possible, and that they are likely to occur sooner rather than later.

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Exhibit B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Boyle et al.

Serial No.: 09/405,032 Group Art Unit No.: 1647

Filed: September 24, 1999 Examiner: DeBerry, R.

For: Osteoprotegerin

Docket No.: A-378CIP2C2

#8
AQJ
10/10/01

DECLARATION OF DR. JACKIE Z. SHENG

I, Jackie Z. Sheng, declare and state as follows:

1. Since 1994, I have been employed by Amgen, Inc., Thousand Oaks, CA and currently hold the position of Research Scientist.

2. From 1992 to 1994 I was Assistant Instructor in the Department of Molecular Genetics at the University of Texas Southwestern Medical School, Dallas, TX. From 1990 to 1992 I was a postdoctoral associate in the Department of Biochemistry at Purdue University, West Lafayette, IN. My research interests are in the areas of gene therapy, molecular virology and molecular biology. Submitted herewith as Attachment No. 1 is a copy of my cirriculum vitae.

3. Since joining Amgen, I have been involved in experiments designed to test various viral vectors to deliver polypeptides for gene therapy applications.

4. I conducted experiments designed to test the activity of OPG upon gene transfer into mice. In experiments that were performed under my direction, I constructed a recombinant adenovirus-based vector for delivery of an OPG fusion polypeptide. The OPG fusion polypeptide comprised amino acids 1-201 of OPG (hereafter OPG[1-201]) as shown in Figure 9C and 9D (SEQ ID NO: 125) of the present application, U.S. Serial No. 09/405,032, fused at its carboxy terminus to the hinge, CH2 and CH3 regions of human IgGγ1 as shown in Ellison et al. (Nuc. Acids Res. 10, 4071-4079 (1982) submitted as Attachment No. 2). DNA encoding the OPG[1-201]-Fc fusion polypeptide was inserted into

the adenoviral shuttle plasmid pACCMVpLpA (described in Gomez-Foiz et al. J. Biol. Chem. 267, 25129-25134 (1992) submitted as Attachment No. 3) as follows. pACCMVpLpA was digested with BamHI, treated with alkaline phosphatase, and digested with SalI. OPG[1-201]-Fc DNA in plasmid pDSRα2 (pDSRα2 is described in WO90/14363) was digested with BamHI and SalI and the DNA fragments ligated. The resulting recombinant plasmid is designated pACCMV-OPG[1-201]-Fc. pACCMV OPG[1-201]-Fc and the large fragment of XbaI-cut plasmid RR5 (RR5 is an Ad-dL309 derivative described in Kopfler et al. Circulation 90, 1319-1327 (1994) submitted as Attachment No. 4) were cotransfected into 293 human embryonic kidney cells (available from the American Type Culture Collection, Manassas, VA under accession no. CRL-1573). Transfected 293 cells were overlaid with DMEM containing 0.65% agarose, 2% fetal calf serum supplemented with penicillin and streptomycin. Recombinant viral plaques containing OPG[1-201]-Fc DNA were isolated, subjected to an additional round of plaque purification, and expanded into stocks containing 5 x 10^{10} plaque forming units (pfu)/ml, using procedures generally described in Attachment No. 4. Recombinant adenovirus were titered by a plaque assay on 293 cells using procedures described in Attachment No. 4.

5. I confirm that the following reagents: OPG DNA, human IgGlγ1 DNA, plasmid pACCMVpLpA, plasmid RR5, and 293 human embryonic kidney cell line were available to one skilled in the art before the priority date of the present application.

6. Based upon information provided to me, ovariectomized and sham-operated mice were prepared as follows. Female C57/BL6 mice aged 8 weeks (Charles River, Wilmington, MA) were anesthetized with isofluorane, shaved and scrubbed with an iodophore solution. An incision was made on one side of the animal and muscle was dissected until fat surrounding the uterus and ovary was exposed. The ovary and uterus were pulled out of the incision and the ovary was clamped at the uterus and removed. The uterus was placed back into the body cavity and the incision closed with skin clips. The same procedure was repeated on the other side of the animals. For sham operated controls, the ovary and uterus were pulled from the incision but the ovary was not removed.

7. In experiments done under my direction, each group of ovariectomized and sham-operated mice (eight animals per group) were injected into the tail vein with one of the

following: about 5 x 10^8 pfu of recombinant adenovirus in PBS carrying OPG[1-201]-Fc DNA; about 5 x 10^8 recombinant adenovirus carrying cDNA encoding β-galactosidase (referred to as AdCMV-βgal in Herz et al. (Proc. Natl. Acad. Sci. USA 90, 2812-2816 (1993), submitted as Attachment No. 5); and a vehicle control of PBS with no virus. 28 days after injection, the mice were euthanized with carbon dioxide.

8. Based upon information provided to me, bone mineral density at two sites, the proximal tibial metaphysis and the lumbar vertebrae (L5), were determined as follows. The fully articulated tibia and femur were removed from the euthanized mice and fixed in 70% ethanol. Bone mineral density was determined at the proximal tibial metaphysis by peripheral quantitative computed tomography (pQCT) (XCT-960M, Norland Medical Systems, Ft Atkinson, WI). Two 0.5mm cross-sections of bone, 1.5mm and 2.0mm from the proximal end of the tibia were analyzed (XMICE 5.2, Stratec, Germany) to determine total and trabecular (defined as the inner most 20% of the bone cross-section) bone mineral density in the metaphysis, and an average value for both cross-sections is reported. A soft tissue separation threshold of 1500 was used to define the boundary of the metaphyseal bone. The total bone mineral density for lumbar vertabrae (L5) was determined in a similar manner. Fully articulated sections of lumbar vertebrae were extracted from the mice and L5 was removed and fixed in 70% ethanol. L5 is designated as the first vertebrae proximal to the iliac crest. A single 1.0mm mid-vertebral cross-section of bone was analyzed. A soft tissue separation threshold of 1500 was used.

9. Based upon information provided to me, the measurements of bone mineral density at the tibial metaphysis and in lumbar vertebrae in ovariectomized and sham-operated mice are shown in Attachment No. 6 (for the tibial metaphysis) and Attachment No. 7 (for the lumbar vertebrae). Ovariectomized mice treated with vehicle (PBS with no virus) had significantly lower bone mineral density at both sites compared to sham-operated mice treated in the same manner. Injection of pACCMV OPG[1-201]-Fc vector into ovarectomized mice increased bone mineral density at both sites compared to sham operated control mice and blocked the loss of bone density in these mice. Injection of the control vector (AdCMV-βgal) did not affect bone

mineral density at either site, although in these mice the effect of ovariectomy on bone mineral density was not statistically significant when compared to sham-operated control mice.

10. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Jackie sheng

Jackie Z. Sheng

9/28/01

Date

Attachment No. 1

Curriculum Vitae

1. Personal

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Job Position	Research Scientist
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2. Education

1984	B.S. (Biology) in Zhongshan University, Guangzhou, Canton, China
1990	Ph.D. (Biochemistry & Molecular Biology) in University of Miami School of Medicine, Miami, Florida.

3. Research and Professional Experience

1994-present	Research Scientist at Amgen Inc. Thousand Oaks, California
1992-1994	Assistant Instructor in Molecular Genetics Department at University of Texas Southwestern Medical Center at Dallas, Texas (with Drs. Michael S. Brown and Joseph L. Goldstein).
1990-1992	Post-doctoral associate in the Department of Biochemistry at Purdue University (with Dr. Jack Dixon and Dr. Harry Charbonneau).
1985-1990	Predoctoral trainee in the Department of Biochemistry & Molecular Biology of University of Miami School of Medicine in Miami (with Dr. Kermit L. Carraway).

4. Honors/Awards

Outstanding Student Award, ZhongShan University, 1981-1984
CUSBEA Fellow (China-U.S. Biochemistry Examination Application, organized by Beijing University, Cornell University and Harvard University), 1984.
Damon Runyon-Walter Winchell Cancer Research Fund Postdoctoral Fellowship, 1992-1993.

5. Publications

1. Spielman, J., Hull, S., **Sheng, Z.**, Kanterman, R., Bright, A. and Carraway, K. L. (1988) "Biosynthesis of a Tumor Cell Surface Sialomucin: Maturation and Effects of Monensin." J. Biol. Chem. 263, 9621-9629.

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3. Hull, S., **Sheng, Z.**, Vanderpuye, O. A., David, C. and Carraway, K. L. (1990) "Isolation and partial characterization of Ascites Sialoglycoprotein-2 (ASGP-2) of the Cell surface sialomucin Complex of 13762 Rat Mammary Adenocarcinoma Cell." Biochem. J. 265, 121-129.

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12. **Sheng, Z.**, Carter, C., Hsieh, A., Deng, H., Capparelli, C., Sean, M., Khoo, H., Bolon, B. and Dunston, C. (1999) " Adenovirus Mediated Osteoprotegerin (OPG) Gene Transfer and Expression in mice" Manuscript in preparation.

13. **Sheng, Z.**, Sean, M., Capparelli, C., Carter, C., Deng, H., Hsieh, A., Bolon, B., Khoo, H., Daris, M. and Dunston, C. (1999) "Long-term Gene expression of Osteoprotegerin Fc Fusion Protein Mediated by Adenovirus and Gene Therapy in a Mouse Osteoporosis Model" Manuscript in preparation.

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ARTICLE

Adenoviral Delivery of Osteoprotegerin Ameliorates Bone Resorption in a Mouse Ovariectomy Model of Osteoporosis

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Osteoprotegerin (OPG) regulates bone resorption by inhibiting osteoclast formation, function, and survival. The current studies employed a mouse ovariectomy (OVX) model of estrogen deficiency to investigate gene therapy with OPG as a means of preventing osteoporosis. Young adult females injected with a recombinant adenoviral (Ad) vector carrying cDNA of either full-length OPG or a fusion protein combining the hOPG ligand-binding domain with the human immunoglobulin constant domain (Ad-hOPG-Fc) developed serum OPG concentrations exceeding the threshold needed for efficacy. However, elevated circulating OPG levels were sustained for up to 18 months only in mice given Ad-hOPG-Fc. Administration of Ad-hOPG-Fc titers between 10^7 and 10^9 pfu yielded dose-dependent increases in serum OPG. Mice subjected to OVX or sham surgery followed by immediate treatment with Ad-hOPG-Fc had significantly more bone volume with reduced osteoclast numbers in axial and appendicular bones after 4 weeks. In contrast, animals given OVX and either a control vector or vehicle had significantly less bone than did comparably treated sham-operated mice. This study demonstrates that a single adenoviral gene transfer can produce persistent high-level OPG expression and shows that gene therapy to provide sustained delivery of OPG may prove useful in treating osteoporosis.

Key Words: **osteoprotegerin; OPG; adenovirus; gene therapy; estrogen deficiency; ovariectomy; disease model; mouse.**

INTRODUCTION

Osteoporosis is a chronic condition chiefly affecting postmenopausal women, in whom the skeleton loses a significant percentage of its mineralized mass and mechanical resiliency, thereby becoming prone to fracture. Approximately 10 million women in America have osteoporosis, while another 17 million are osteopenic and at risk of developing the disease (1). The loss of estrogen at menopause is a major contributor to disease pathogenesis because this hormone is a principal negative regulator of osteoclast activity (2–4), and osteoclasts are the chief effector cells responsible for bone remodeling in osteoporosis (5). Thus, based on this pathway effective mechanisms for treating osteoporosis include estrogen replacement or osteoclast inhibition. Two therapies currently exist for this condition. Bisphosphonates (e.g., pamidronate) adsorb to bone mineral and physicochemically prevent remodeling by osteoclasts. However, these agents have low oral availability and may cause significant gastrointestinal irritation (6) and acute phase reactions (7). In contrast, selective estrogen receptor modulators such as raloxifene replace estrogen and thereby down-regulate osteoclast activity. Unfortunately, these molecules may be less efficacious than newer bisphosphonates at preventing bone loss and may cause "hot flashes" (8). Thus, a substantial opportunity exists for new agents that could prevent bone remodeling with fewer side effects.

Osteoprotegerin (OPG), a novel soluble protein of the tumor necrosis receptor family, offers considerable promise as a new modality for treating osteoporosis based on its novel mechanism. OPG inhibits osteoclast formation,

This study was presented in part as abstracts at the 18th Annual Meeting of the Society of Toxicologic Pathologists, Washington, DC, June 14–17, 1999, and at the Sun Valley Hard Tissue Workshop, pQCT Users Meeting, Sun Valley, Utah, August 7–9, 1999.

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function, and survival by preventing the binding of RANK ligand (RANKL; also known as OPG ligand) to RANK (9). RANK (receptor activator of NF-κB) is a membrane-bound protein of the TNF receptor family found on chondrocytes, osteoclast precursors, and mature osteoclasts (10–17). Several lines of inquiry indicate that altered homeostasis in the OPG/RANKL/RANK signaling pathway may contribute to the osteoporosis that results from menopause-induced estrogen deficiency (18). Animals with null mutations of the *OPG* gene exhibit severe osteoporosis (20, 21), while transgenic mice that express supraphysiological levels of OPG develop profound osteopetrosis (10). Mice with ablated *RANKL* and *RANK* genes exhibit osteopetrosis as well (22, 23). Administration of recombinant OPG prevents bone loss in rat models of ovariectomy-associated estrogen deficiency (10) and adjuvant arthritis (24). Recently, results of the first human trial with OPG supported its potential as a therapeutic agent for osteoporosis (25).

Although OPG can be delivered via protein injection, osteoporosis is a chronic disease that will require decades of therapy and thus repetitive injections. It would be desirable to develop methods for treatment which require less frequent injection. One avenue to provide sustained treatment from each injection would be through a gene therapy method. In the current studies, the ability of OPG to positively impact the progress of bone-destructive conditions was assessed by examining the extent of bone protection provided by adenovirus vectors designed to deliver OPG. For this purpose, a mouse ovariectomy (OVX) model of estrogen deficiency and osteoporosis was used as the bioassay system. The present data show that it is feasible to provide long-lasting expression of OPG at bone-protective levels using a gene therapy approach.

Materials and Methods

Construction of replication-defective adenoviral (Ad) vectors. Methods for the construction of replication-deficient Ad vectors have been described (29). Briefly, cDNA of full-length human OPG (hOPG), murine OPG (mOPG), or a fusion protein (hOPG-Fc) combining the hOPG ligand-binding domain with the constant domain of human IgG1 (10) was cloned into the shuttle plasmid pACCMVpLpA (30). Individual shuttle plasmids were cotransfected with the large fragment of *Xba*I-cut pAd-dL309 derivative into 293 cells. Appropriate recombinant plaques containing hOPG, mOPG, or hOPG-Fc were isolated, propagated, and titrated as described (29). Adenovirus containing the β-galactosidase gene (β-gal) was used as a control (31).

Molecular procedures. For Southern blot analysis, mouse livers were homogenized and total cellular DNA was isolated. A 10-μg aliquot of DNA was digested with *Kpn*I, separated by 0.8% agarose gel electrophoresis, and transferred to nylon membrane (32). ^{32}P-labeled full-length hOPG cDNA or a fragment isolated from the adenovirus type 5 (coordinate nt 1–453) was used as probes to detect hOPG or adenovirus sequences, respectively.

Serum concentrations of OPG were assessed using proprietary primary IgG directed against human OPG (10). For Western blot analysis (33), serum was fractionated by SDS–PAGE (4–20% gels) and transferred to nitrocellulose membranes. Blots were probed with rabbit polyclonal anti-OPG antibody (0.1 μg/ml) followed by horseradish peroxidase (HRP)-conjugated donkey polyclonal anti-rabbit IgG (1:2000) and then detected by enhanced chemiluminescence (Amersham Life Sciences) according to the manufacturer's instructions. For ELISA (33), 96-well plates were coated with mouse monoclonal anti-human OPG (0.3 μg/well) overnight at 4°C, and then incubated overnight at 4°C with blocking solution (10 mM Tris, pH 7.5; 145 mM NaCl; 2% BSA; 2% glycerol; 10% sucrose). Wells were incubated at room temperature with dilute serum (50 μl) for 1.5 h followed by HRP-conjugated rabbit anti-mouse IgG (1:35,000, 100 μl) for 1 h. The detection limit for ELISA was 0.2 ng/ml.

Animals. Female adult C57BL/6 or CD-F1 mice (Charles River, Wilmington, MA) were randomly assigned to treatment groups (n = 5 to 8 per group) and were injected via tail vein with 100 μl of vehicle (145 mM NaCl, 1 mM $MgCl_2$, 1 mM KCl, 10 mM Tris, pH 7.6, and 10% glycerol) containing 0 or the indicated doses of recombinant adenoviruses carrying β-gal, mOPG, hOPG, or hOPG-Fc cDNA. Blood was collected from tail veins on indicated days. OPG expression was visualized by Western blot analyses before being pooled for ELISA to quantify the protein level.

Efficacy bioassay. Bilateral OVX in mice is a standard method to evaluate agents that may ameliorate bone loss associated with estrogen deficiency (35). On Study Day −1, CDF1 mice were anesthetized with isoflurane to allow exposure of the ovaries; the gonads were removed in "OVX" groups but only manipulated in "Sham" cohorts. On Day 0, mice were injected with 0 or 5×10^8 pfu of either Ad-hOPG-Fc or Ad-β-gal. Bone density was estimated by acquiring whole-body radiographs for 49 s at 0.3 mA and 55 KVP on Day 14 after injection using a benchtop radiography unit (Model 43855A; Faxitron X-ray Corp., Buffalo Grove, IL). At Day 28, animals were necropsied to evaluate routine hematologic and clinical chemistry panels (34) as well as the morphology of bone (tibia; for OPG effects) and liver (for Ad toxicity). Lesion severity was graded as absent, minimal, mild, moderate, or marked. A serial section of tibia was also stained for the osteoclast marker tartrate-resistant acid phosphatase (TRAP) as previously described (36).

Bone mineral density (BMD) was quantified in ethanol-fixed, undecalcified bones by peripheral quantitative computed tomography analysis (XCT-960M tomograph; Norland Medical Systems, Fort Atkinson, WI; XMICE 5.2 statistical software; Stratec, Pforzheim, Germany). In the tibia, BMD was acquired for "total" (i.e., the entire bone) and "trabecular" (i.e., the inner 20% of bone mass) zones by averaging values from two 1.25-mm sections, located 0.5 mm apart, taken 1.5 mm distal to the proximal articular surface. In contrast, BMD in the "cortical" zone of the tibia was measured in one 1.25-mm section located 4.0 mm distal to the articular surface. For the fifth lumbar vertebra, BMD for all three zones were defined in one 1.25-mm midvertebral cross section. For both bones, the total and trabecular regions were differentiated from soft tissue using a separation threshold of 1500, while the cortical zone was delineated using a threshold of 2000.

Data analysis. Data were assessed using JMP statistical software (v. 3.2.1; SAS Institute, Cary, NC).

Results and Discussion

Massive and Persistent Expression Occurred with hOPG-Fc

The current studies were designed as an acute "proof-of-concept" system for using gene therapy as a bone-protective regimen. A pilot experiment was performed to assess the OPG expression profile mediated by adenoviruses carrying cDNA of either full-length OPG (mouse or human) and a fusion construct of hOPG-Fc. Comparison of these homologues would allow us to choose the best molecule to proceed with in the studies. Basal OPG levels in mice treated with vehicle or Ad-β-gal were below the detection limit of the ELISA (0.2 ng/ml). In contrast, all animals injected with any Ad-OPG construct (5×10^8 pfu) exhibited appreciable elevations in serum OPG (at least 2 μg/ml) within 1 day of inoculation, with peak production evident by day 4 (Fig. 1). At both these times, the mean serum OPG concentration resulting from treatment with Ad-hOPG-Fc was approximately 10- and 100-fold higher,

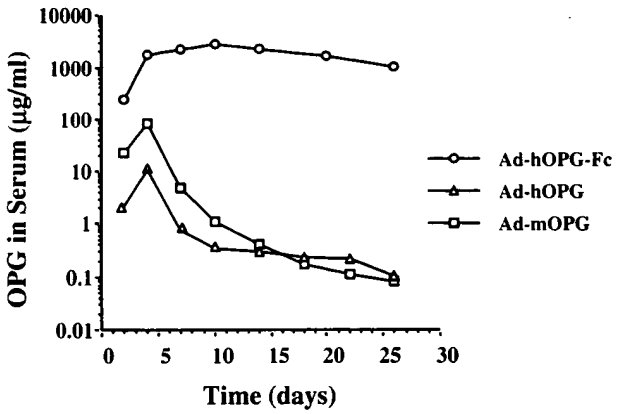

FIG. 1. Production of hOPG-Fc was higher and more sustained than that of hOPG or mOPG after a single intravenous injection of adenovirus. Four-week-old C57BL/6 mice were given recombinant adenovirus (5×10^8 pfu) carrying cDNAs of hOPG-Fc fusion protein (Ad-hOPG-Fc), full-length hOPG (Ad-hOPG), or full-length mOPG (Ad-mOPG) protein. Serum OPG concentrations were measured by ELISA on Days 1, 4, 7, 10, 14, 18, 22, and 26. Circulating OPG was not detected in control mice injected with either vehicle or adenovirus containing β-galactosidase (Ad-β-gal). These data were used to select the optimal molecule for further experiments.

respectively, than that produced by Ad-mOPG and Ad-hOPG (Fig. 1). In fact, from day 4, hOPG-Fc was a major protein fraction in the sera, second only to albumin. The circulating OPG levels in mice given Ad-mOPG or Ad-hOPG exhibited a 10-fold reduction from the peak 7 days after viral injection and the kinetics of expression from the treatment of these two viruses were very similar (Fig. 1). In contrast, OPG concentrations induced by Ad-hOPG-Fc were maintained at about 1 mg/ml for the entire 1-month pilot study (Fig. 1). Possible explanations for these data are that the presence of the Fc domain in the fusion molecule or the absence of the native protein's heparin-binding domain imparted a greatly extended half-life to hOPG-Fc relative to those of the native forms (10). Other potential reasons for the selective persistence of hOPG-Fc could be that its mRNA was more stable or that loss of antigenic OPG epitopes in the fusion protein reduced its antigenicity. Also, the possibility that some feature specific to the Ad-hOPG-Fc vector resulted in extended persistence of the viral construct in transduced cells cannot be ruled out. None of these possibilities was tested in the current studies. Since Ad-hOPG-Fc resulted in both the highest and the most sustained levels of circulating OPG, all remaining experiments in the present proof-of-concept studies were performed using only this construct.

hOPG-Fc Expression and Vector-Associated Toxicity Are Dose Dependent

A dose–response experiment was performed to determine an Ad-hOPG-Fc titer providing efficient sustained expression of OPG while inducing minimal toxicity. As shown in Fig. 2, injection of between 2×10^7 and 6×10^7 pfu of Ad-hOPG-Fc resulted in peak hOPG-Fc concentrations of 2.6 and 4.4 μg/ml, respectively. The OPG levels declined almost to baseline after only 2 weeks for the lowest titer but were modestly increased (3.6 ng/ml) above control levels (<0.2 ng/ml) after 32 days for the 6×10^7 pfu dose. Neither of these low titers resulted in a sustained increase in bone density (i.e., efficacy) or elevated serum activities of hepatocyte enzymes (i.e., toxicity) (data not shown). In contrast, Ad-hOPG-Fc titers above 2×10^8 resulted in greatly augmented protein production (Fig. 2), with peak levels of 440 μg/ml at 2×10^8 pfu, 4040 μg/ml at 6×10^8 pfu, and 5680 μg/ml at 2×10^9 pfu. Circulating hOPG-Fc dropped 55-fold to 8 μg/ml for the 2×10^8 pfu dose after 1 month, while levels for the two highest doses were maintained near the mg/ml level. All three of the higher Ad-hOPG-Fc titers appreciably augmented bone density on radiographs throughout the duration of the study (Fig. 3). Toxicity was assessed by measuring serum activities of hepatocyte enzymes (34). Serum concentrations of hepatocyte enzymes were not significantly affected by injection of Ad-hOPG-Fc at 2×10^7, 6×10^7, and 2×10^8 pfu (data not shown). Enzyme levels exhibited a modest (3-fold) elevation in mice given 6×10^8 pfu. In contrast, significant hepatotoxicity was evident after injection of 2×10^9 pfu as indicated by greater than 5-fold elevations in serum enzyme activities in conjunction with clinical jaundice (data not shown). Based on the expression level and clinical chemistry analysis of serum, we chose 5×10^8 pfu for further studies to evaluate long-term expression of hOPG-Fc in mice and its effect in a biologically relevant model.

Efficacious Level of hOPG-Fc Was Maintained for at Least 18 Months after a Single Ad-hOPG-Fc Injection

Long-term expression of hOPG-Fc mediated by Ad-hOPG-Fc was performed in C57BL/6 mice. As demonstrated in Fig. 4, injection of 5×10^8 pfu of Ad-hOPG-Fc

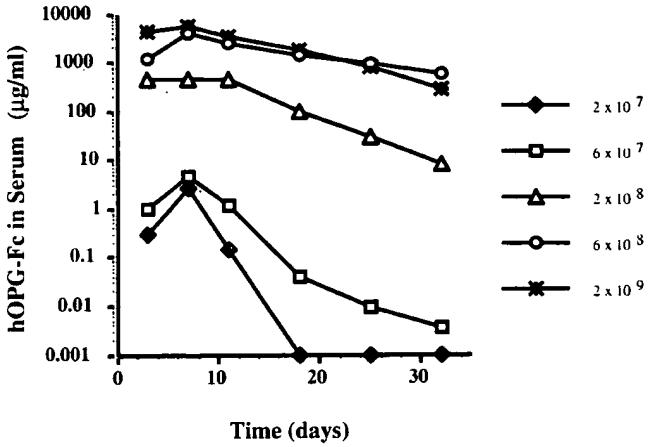

FIG. 2. Circulating hOPG-Fc levels were correlated to viral titer. Four-week-old C57BL/6 mice were intravenously injected with Ad-hOPG-Fc at various titers (range, 2×10^7 to 2×10^9 pfu). Serum OPG concentrations were measured by ELISA on Days 3, 7, 11, 18, 25, and 32. Animals given Ad-β-gal had undetectable OPG levels.

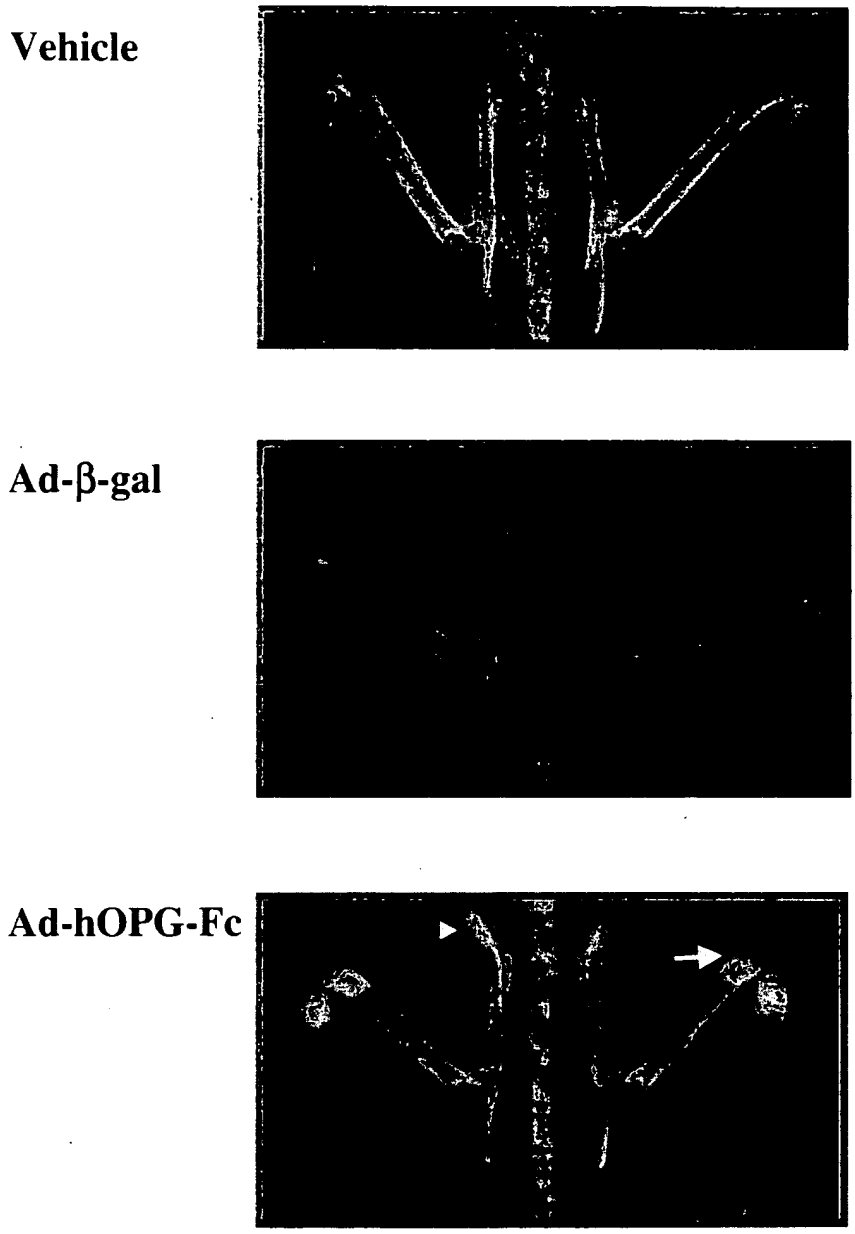

FIG. 3. Expression of hOPG-Fc was sufficient to increase bone density. Four-week-old C57BL/6 mice were injected with Ad-hOPG-Fc at various titers (range, 2×10^7 to 2×10^9 pfu). Efficacy was assessed on Study Day14 by evaluating skeletal opacity in whole-body radiographs. Note the increased density of the metaphyses of long bones (arrow) as well as the components of the pelvis (arrowhead) and vertebrae.

resulted in high levels of circulating hOPG-Fc (250 μg/ml) within 1 day of viral injection, with peak production of 3 mg/ml evident by day 10. In fact, at the peak of expression, hOPG-Fc stayed in serum as a major protein, second to serum albumin (Fig. 4B). The OPG levels declined in a biphasic manner. A dramatic reduction (60-fold) of hOPG-Fc expression occurred in the first 2 months after injection of the vector, while a slow additional 10-fold decrease was observed after another 5 months (Fig. 4A). At Day 7 and Day 150 after injection, high-molecular-weight DNA was isolated from livers of the mice injected with vehicle, control virus, or Ad-hOPG-Fc and probed with ^{32}P-labeled hOPG to assess if hOPG-Fc expression levels were associated with sustained presence of hOPG-Fc cDNA. Surprisingly, abundant hOPG-Fc DNA was detected in the livers of mice 5 months after treatment with Ad-hOPG-Fc (Fig. 4C). Clearly, serum hOPG-Fc concentrations measured by ELISA declined much more rapidly (200-fold) than did the hOPG-Fc DNA levels (less than 10-fold) detected by Southern analysis, suggesting a possible promoter silencing effect in the livers. It is also possible that immune-competent mice developed antibodies to the fusion protein. In a separate experiment, long-term hOPG-Fc expression was followed up to 18

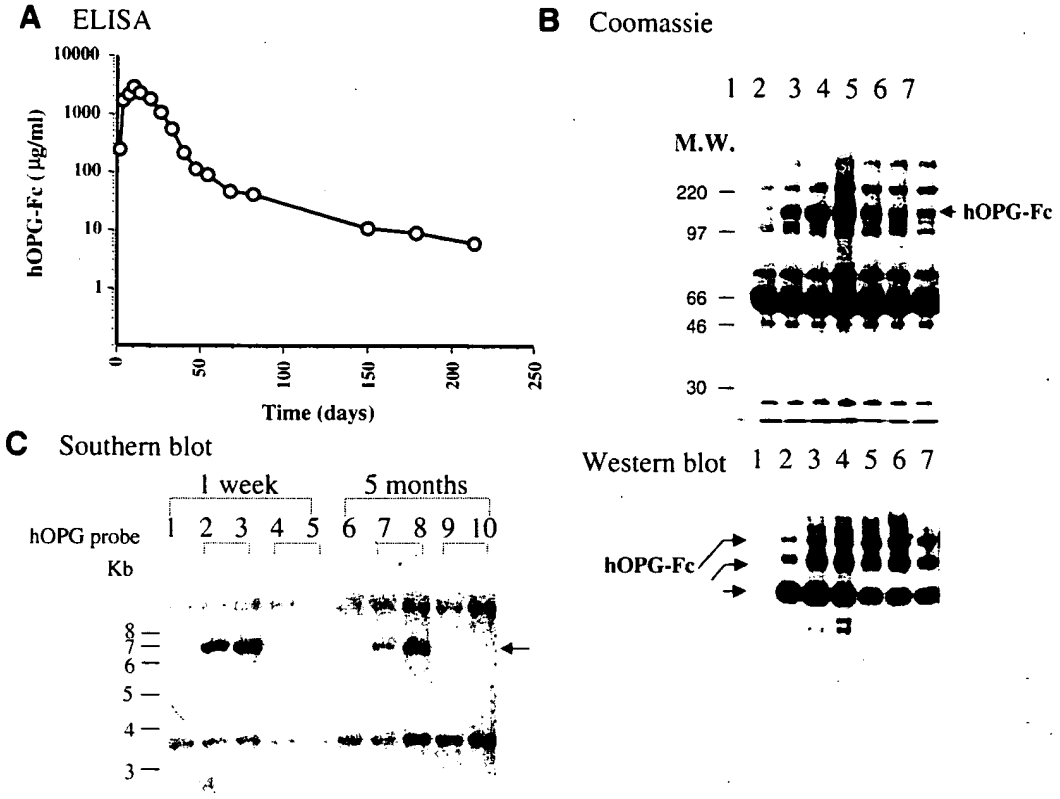

FIG. 4. A single intravenous injection of Ad-hOPG-Fc resulted in sustained circulating levels of hOPG-Fc for months. Four-week-old C57BL/6 mice were given Ad-hOPG-Fc (5×10^8 pfu). Serum OPG levels were measured by ELISA on Days 2, 4, 7, 10, 14, 18, 21, 26, 40, 48, 54, 68, 82, 150, 179, and 218 (A). Coomassie blue staining showed that hOPG-Fc (arrow) was second only to albumin as a major serum protein (B, top); corresponding Western blot analysis is shown in B, bottom. Arrows denote dimer and multimers of hOPG-Fc. Lanes 1–7 correspond to Days 0, 4, 7, 10, 14, 18, and 21 after injection. (C) Southern blot analysis of liver DNA isolated from individual mice 7 and 150 days after injection. Lanes 1 and 6, vehicle control; lanes 2, 3 and 7, 8, Ad-hOPG-Fc; lanes 4, 5 and 9, 10, Ad-β-gal. Arrow denotes DNA fragment positive for hOPG-specific probe. The lower size fragment is nonspecific but provides an internal control for loading.

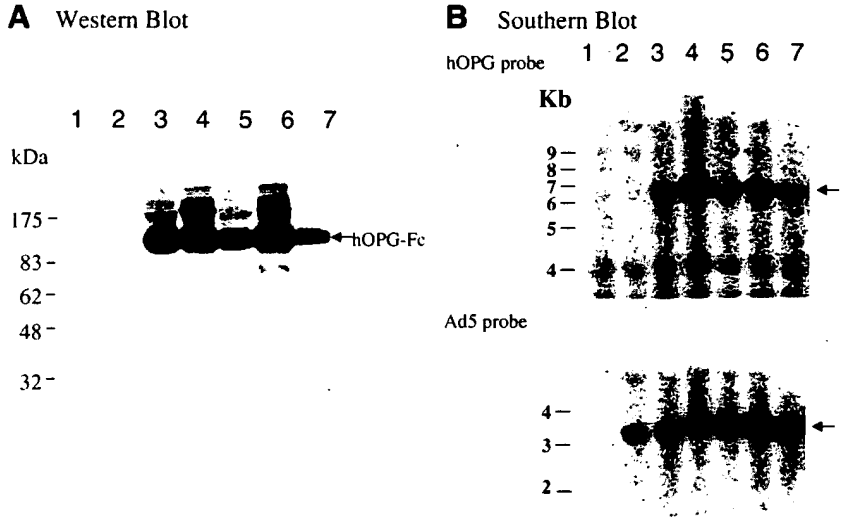

FIG. 5. Long-term expression of serum hOPG-Fc is associated with sustained presence of hOPG-Fc cDNA in liver. Eighteen months after viral injection, expression of hOPG-Fc in serum (A, Western blot analysis) was compared to hOPG-Fc DNA level (B, Southern blot analysis). Lanes correspond to seven individual animals, as follows: 1, vehicle control; 2, Ad-β-gal control; 3–7, Ad-hOPG-Fc. Arrows denote DNA fragment positive for either hOPG or adenovirus DNA.

TABLE 1

Sustained hOPG-Fc Expression Leads to Enhanced Bone Mineral Density in an Osteoporosis Model

			Tibia			Lumbar vertebrae (L5)		
			Metaphysis		Diaphysis			
Group (n = 8)	Treatment	Surgical procedure	Total density	Trabecular density	Cortical density	Total density	Trabecular density	Cortical density
1	Ad-β-gal	Sham	423.6	384.2	661.9	305.0	115.3	474.9
			37.8	61.6	19.8	15.1	12.9	7.3
2	Ad-OPG	Sham	595.7*	585.3*	659.7	360.8*	136.2*	510.1*
			54.4	71.3	44.2	17.8	32.0	13.5
3	Vehicle	Sham	425.4	338.6	651.5	325.3	116.0	482.1
			27.8	44.3	43.3	27.6	47.5	11.1
4	Ad-β-gal	OVX	408.0	365.9	672.6	307.0	117.0	472.5
			20.0	28.4	35.9	16.3	15.6	12.9
5	Ad-OPG	OVX	576.8*	584.1*	674.4	378.2*	170.8*	512.5*
			52.1	71.5	55.4	19.4	30.7	14.8
6	Vehicle	OVX	386.0	303.3	659.9	295.6	117.0	464.2
			24.1	29.7	30.7	14.1	10.7	10.0

Note. Values represent means (upper values) and standard deviations (lower values).
* Significant difference from operation-matched, vehicle-treated mice.

months. At the end of the study, average circulating hOPG-Fc concentration in five individual mice was still at μg/ml level (2.5 μg/ml) as measured by ELISA, and the presence of DNA derived from hOPG-Fc and adenovirus DNA was still evident at the end of the study (Fig. 5). This suggested that extended gene expression from hOPG-Fc cDNA as well as the long half-life contributes to the sustained presence of hOPG-Fc in serum.

OPG Expression Mediated by Ad-hOPG-Fc Enhances Bone Density in OVX Model

Ovariectomy in mice is a standard model system to evaluate bioactive molecules that may ameliorate bone loss associated with estrogen deficiency (35). Four weeks after OVX, two physiological changes confirmed that the procedure had successfully induced a degree of estrogen

TABLE 2

Systemic Physiologic Changes 28 Days after Adenoviral Injection (5×10^8 pfu)[a]

Group (n = 8)	Treatment	Surgical procedure	Body weight	Liver %BW	Spleen %BW	LDH	AST	ALT
1	Ad-β-gal	Sham	21.9	**5.69***	**0.56***	1117*	235*	223*
			0.8	0.22	0.06	143	85	87
2	Ad-OPG	Sham	21.8	**5.72***	**0.68***	**972**	**202**	243*
			0.7	0.36	0.07	406	157	292
3	Vehicle	Sham	21.2	4.74	0.42	475	70	27
			1.0	0.30	0.08	96	12	7
4	Ad-β-gal	OVX	24.0[b]	**5.96***	**0.63***	**1017**	273*	311*
			0.9	0.21	0.08	276	157	108
5	Ad-OPG	OVX	23.8[b]	**5.62***	**0.74***	**870**	**178**	173*
			1.8	0.22	0.08	503	102	110
6	Vehicle	OVX	24.3[b]	4.58	0.42	433	81	25
			1.5	0.27	0.07	142	45	6

Note. Bold values denote apparent but not statistically significant difference from operation-matched, vehicle-treated mice. Abbreviations: LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase. Values represent means (upper values) and standard deviations (lower values).
[a] Weights in grams, serum enzyme levels in units per liter.
[b] Significant difference relative to treatment-matched, sham-operated mice.
* Significant difference from operation-matched, vehicle-treated mice.

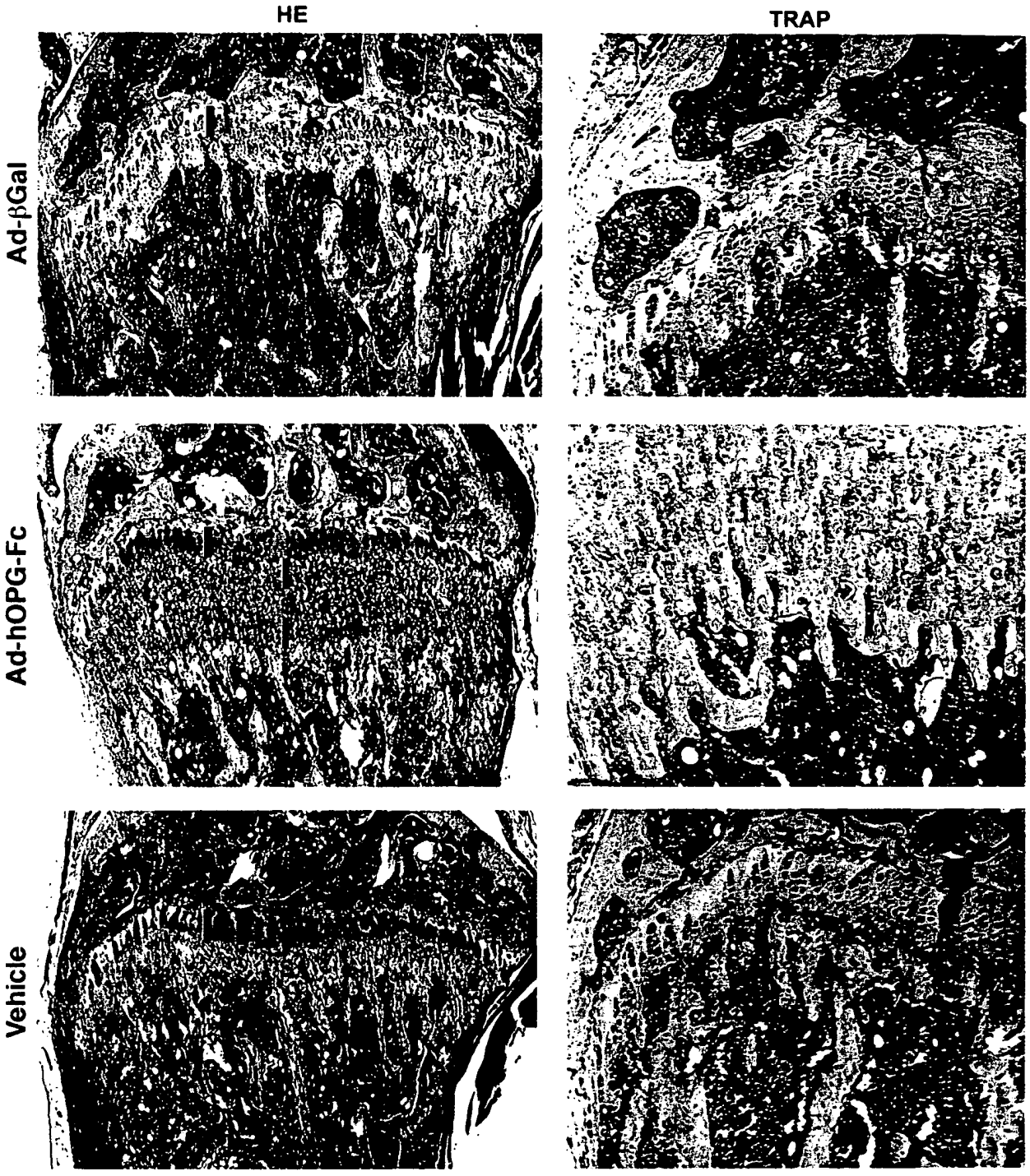

FIG. 6. Expression of hOPG-Fc enhanced bone density by preventing bone resorption. Administration of Ad-hOPG-Fc (5×10^8 pfu) resulted in expansion of the primary spongiosa (S) in the metaphysis of long bones (left) by eliminating osteoclasts (shown on the right as red-stained cells lining the growth plate and bony trabeculae). Sagittal section through the proximal tibia. Bar, normal width of growth plate. Left: hematoxylin and eosin, original magnification 60×. Right: tartrate-resistant acid phosphatase, original magnification 100×.

deficiency capable of initiating skeletal loss. First and foremost, OVX control mice (treated with either vehicle or Ad-β-gal) had bone densities 5 to 10% lower than sham-operated animals given the same treatment (Table 1). Whether this deficit resulted from a loss of previously formed bone, an inability to add new bone layers, or both cannot be determined from these data. Second, all OVX mice had slightly higher body weights (Table 2) and much smaller uterine horns (data not shown) than did sham-operated animals.

While rapid skeletal growth renders the evaluation of a "bone-protective" effect difficult in this rodent OVX model, the present data clearly demonstrated that overexpression of hOPG-Fc greatly augmented bone density throughout the skeleton (comparable to changes shown in Fig. 3), beginning as early as 7 days after Ad-hOPG-Fc injection (data not shown). These changes were noted in both sham-operated and OVX mice; increases were slightly greater for the OVX animals. Relative to vehicle-treated animals, significant enhancement ranging up to 50% was observed in OPG-treated mice in the proximal tibia and vertebra for both the cortical and the trabecular compartments (Table 1). Histopathological analysis at 1 month demonstrated that this increase resulted from a two- to threefold volume increase for the primary spongiosa subjacent to the growth plate (Fig. 6). However, based on this finding alone, it cannot be determined whether OVX mice lost bone or simply stopped adding new layers. The number of TRAP-positive osteoclasts was greatly reduced in mice given Ad-hOPG-Fc relative to animals treated with the control vector (Ad-β-gal) or vehicle (Fig. 6).

While sustained production of hOPG-Fc enhancing bone density in a biologically relevant model of OVX offers considerable promise, the induction of minimal but identifiable liver toxicity by a single injection of Ad-hOPG-Fc has significant implications on the limitation of using first-generation adenoviral vectors to treat chronic conditions. For example, relative liver weights of mice given Ad-β-gal or Ad-hOPG-Fc were significantly higher than those of vehicle-treated animals (Table 2). In all mice injected with either vector, serum activities of one or more hepatocyte enzymes were significantly elevated (Table 2). Histopathologic examination revealed that all these mice had diffuse but minimal hepatocyte apoptosis and lymphocyte infiltration (likely cytotoxic T cells (38, 39)) throughout the liver (data not shown). Other drawbacks with this treatment were that prolonged expression of hOPG-Fc was observed only in those mice treated with a high titer of Ad-hOPG-Fc (thus more toxicity) and that a desirable, consistent level of hOPG-Fc over an extended period of time could not be maintained. Thus it is critical to develop gene therapy methods that will cause significantly fewer adverse effects while mediating effective, sustained gene expression. Another important aspect that needs to be addressed in future studies is whether the OPG expression mediated by gene therapy approaches can successfully treat existing osteoporosis in addition to preventing bone density loss. Studies using a more suitable vector are under way to achieve a more realistic, improved gene therapy approach.

SUMMARY

Regular subcutaneous injection of recombinant human OPG has been shown to ameliorate bone loss in rodent models of adjuvant arthritis (24), bone metastases (40), and estrogen deficiency, including OVX (10). Administration of OPG protein has shown promise of treating osteoporosis in nonhuman primates (37) and in humans (25). The circulating level of OPG required to inhibit bone turnover in adult primates (cynomolgus monkeys) is 30 to 50 ng/ml (37). In current studies, we have established a gene therapy model of preventing osteoporosis by viral delivery of OPG gene. The data are significant because they show that a single treatment with an OPG-expressing gene therapy vector can provide sustained and efficacious levels of circulating OPG that enhance bone mineral density and reduce osteoclast numbers for an extended period of time. Even though constructs containing native hOPG and mOPG provided robust protein expression and led to a short-term increase in bone density in treated mice, the gene expression was transient and the Ad-hOPG and Ad-mOPG systems did not result in a sustained biologic effect. In contrast, the construct carrying hOPG-Fc provided 10- and 100-fold, respectively, more protein than those elaborated by Ad-mOPG and Ad-hOPG on Day 4 after injection. More importantly, treatment with Ad-hOPG-Fc gave stable OPG expression at therapeutic levels for over 18 months. Most significantly, in a biologically relevant model of osteoporosis, OPG expression mediated by a gene transfer vehicle exhibited robust positive effects in preventing bone density loss induced by ovariectomy. These studies clearly demonstrated that a single injection of a recombinant adenovirus carrying the hOPG-Fc gene can effectively ameliorate osteoporosis associated with estrogen deficiency for an extended period. Thus, the present data provide compelling evidence for continued development of gene therapy methods to deliver OPG (or other biological response modifiers) to patients with chronic bone-damaging conditions such as osteoporosis.

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Attachment No. 2

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The nucleotide sequence of a human immunoglobulin $C_{\gamma 1}$ gene

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ABSTRACT

We report the nucleotide sequence of a gene encoding the constant region of a human immunoglobulin γ1 heavy chain ($C_{\gamma 1}$). A comparison of this sequence with those of the $C_{\gamma 2}$ and $C_{\gamma 4}$ genes reveals that these three human C_{γ} genes share considerable homology in both coding and noncoding regions. The nucleotide sequence differences indicate that these genes diverged from one another approximately 6-8 million years ago. An examination of hinge exons shows that these coding regions have evolved more rapidly than any other areas of the C_{γ} genes in terms of both base substitution and deletion/insertion events. Coding sequence diversity also is observed in areas of C_H domains which border the hinge.

INTRODUCTION

Immunoglobulin G (IgG) molecules in humans are divided into four subclasses based on the presence of particular gamma heavy chain constant regions (C_{γ}). These C_{γ} regions ($C_{\gamma 1}$, $C_{\gamma 2}$, $C_{\gamma 3}$, and $C_{\gamma 4}$) are encoded by distinct germline genes (1) which are presumed to be the products of gene duplication of an ancestral C_{γ} gene. Several species of mammals have been shown to possess IgG subclasses, although the number of subclasses varies for different species. For example, both humans and mice have four subclasses, while guinea pigs have two and rabbits have only a single type of IgG. Structural studies at the protein and DNA level have been carried out with several species, and have shown that the homology relationships within the C_{γ} gene families are different for different mammals (2-9). For example, human C_{γ} protein regions are over 90% homologous (2-5), while mouse C_{γ} genes share significantly less homology (70-80% at the nucleotide level (6-8)). Moreover, cross-species comparisons reveal no clear correspondence between individual human and m use genes. These intra- and interspecies homology relationships, as well as the different numbers of C_{γ} genes found in different mammals, indicate that the various mammalian C_{γ} gene families have evolved quite differently since the time of mammalian speciation.

We are interested in studying structural features of human C_{γ} genes in order to gain insights int the evolution of the human C_{γ} gene family. We have previously

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characterized the $C_{\gamma 2}$ and $C_{\gamma 4}$ genes (10,11). In this paper we report the complete nucleotide sequence of a $C_{\gamma 1}$ gene and compare the three human C_{γ} sequences.

MATERIALS AND METHODS

Materials

The human fetal liver DNA library was obtained from T. Maniatis. Sources of nucleic acid enzymes, reagents for DNA sequencing, E. coli strain JM101, and the phage M13mp2 were those described by Steinmetz et al. (12).

Isolation and restriction mapping of a human $C_{\gamma 1}$ genomic clone

Screening of a human fetal liver DNA library cloned in lambda Charon 4A bacteriophage with a human $C_{\gamma 3}$ cDNA probe was done as previously described (10). Mapping of restriction sites for the enzymes Eco RI, Bam HI, Hind III, Xba I, Bgl II, and Pvu II was done by analysis of single and double digests with these enzymes.

Subcloning and DNA sequence analysis

The 3.0 kb Hind III-Pvu II fragment of clone HG3A (see Fig. 1) was digested separately with frequent-cutting restriction enzymes and the products were subcloned into the phage M13mp2 as described (11). Subclones were chosen for sequence analysis following screening of plaques with a labelled genomic fragment containing a full-length $C_{\gamma 4}$ gene (see refs. 10 and 11). DNA sequencing of individual subclones was carried out as described (11). The composite C_{γ} DNA sequence was determined either by overlaps of sequenced regions or by homology of the translated DNA sequence to existing sequence data for a human immunoglobulin γ1 protein (2).

RESULTS AND DISCUSSION

The primary structure of a human $C_{\gamma 1}$ gene

We have previously described the isolation of human C_{γ} genes from a recombinant phage library of fetal liver DNA, using as hybridization probe a cDNA encoding part of a $C_{\gamma 3}$ gene (10). One of these clones, HG3A, is shown diagrammatically in Fig. 1. The restriction map of this clone indicated that it is a distinct species from the clones shown to contain $C_{\gamma 2}$ and $C_{\gamma 4}$ genes (10,11). A 2.6 kb region from clone HG3A containing sequences hybridizing to a full-length $C_{\gamma 4}$ gene was sequenced by the dideoxynucleotide chain-termination method in the phage M13mp2. The sequence obtained is shown in Fig. 2, where we see that the gene has the same basic exon-intron organization that has been previously observed for both human (10,11) and mouse (6-8) C_{γ} genes. The three C_H domains and the hinge segment of the polypeptide are encoded in individual exons that are separated from one another by introns, the largest one lying between the C_H1 and hinge exons. The predicted amino acid residues are listed above the corresponding codons in Fig. 2, and

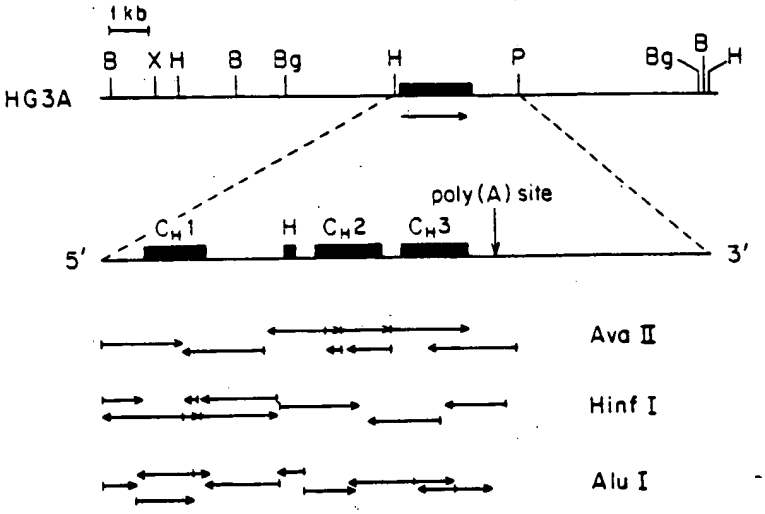


Figure 1. Restriction map and sequencing strategy of a cloned human DNA fragment containing a $C_{\gamma 1}$ gene. Letters on the top line refer to cleavage sites for the foll wing restriction enzymes: B, Bam HI; H, Hind III; Bg, Bgl II; P, Pvu II; X, Xba I. Only the indicated Pvu II site was mapped, although this enzyme also cuts in other places in the clone. The arrow under the solid block indicates the direction of transcription. The dashed lines lead to an enlarged view of the region which was sequ nced. Individual exons are shown here as solid blocks, whereas introns are not indicated at the top of the Figure. The arrowed lines represent the extent and direction of sequence determinations of individual subclones generated using the indicated enzymes.

a comparison of this protein sequence with that of the heavy chains of the two human IgG1 molecules Eu (2) and Nie (13) lead to an unambiguous designation of the cloned sequence as a $C_{\gamma 1}$ gene. Except for differences in amide assignments of several residues, the encoded protein sequence differs from the Eu sequence at just three of 329 compared residues, and only one difference is seen in a comparison with the Nie heavy chain. These differences do not include the lysine encoded at the C-terminus of the C_H3 domain, which has been observed in mouse (6-8) and human (10,11) C_γ genes but does not appear in the mature polypeptides. Table 1 compares the lengths of the exons and introns of the human and mouse C_γ genes that have been sequenced to date. Although some variation is seen in the lengths of noncoding regions and hinge exons, the overall organization of the C_γ genes is conserved in humans and mice.

Antigenic determinants have been found on human IgG molecules which can serve as genetic markers for C_H regions (14). Some of these allelic variants, called allotypes, have be n correlated with specifi amino acid residues in the heavy chains

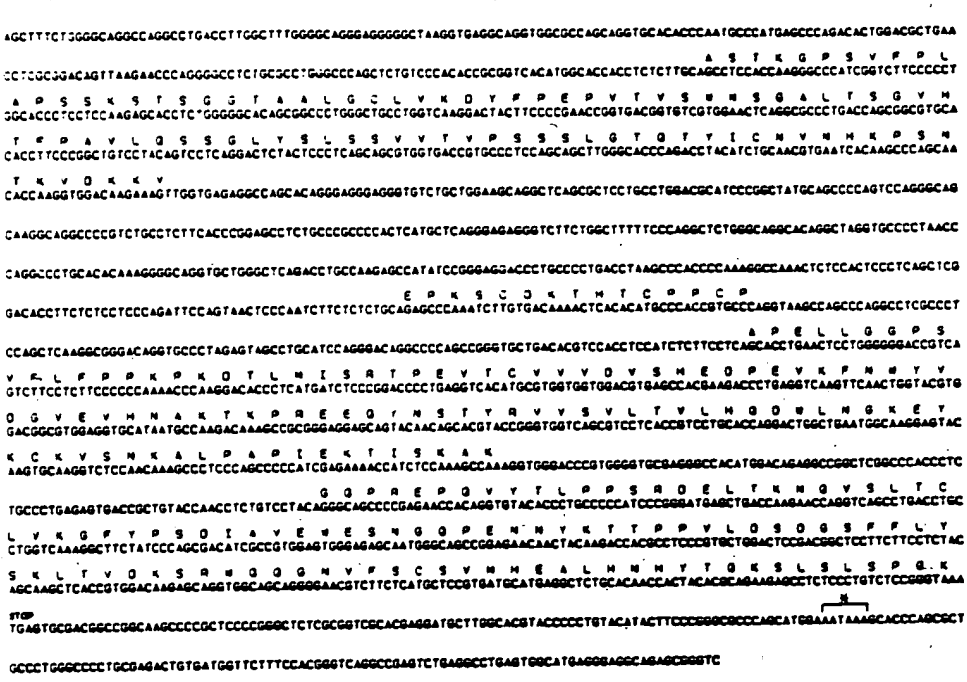


Figure 2. The nucleotide sequence of a human $C_{\gamma 1}$ gene and its corresponding protein sequence. The sequence of the mRNA synonymous strand is listed 5' to 3'. Amino acids predicted by the DNA sequences are listed in one-letter code above the respective codons. "Stop" indicates the termination codon UGA. The presumptive poly(A) addition signal sequence is marked by an asterisk.

(15). We find that the discrepant residues in the Eu heavy chain and the encoded polypeptide reported here can be correlated with certain of these allotypic markers. The lysine encoded at position 97 of the C_H1 domain (Fig. 2) correlates with the Gm (17) determinant, while the arginine at the corresponding place in the Eu heavy chain is associated with the Gm (3) marker. Similarly, the asp-glu-leu sequence at positions 16-18 of the C_H3 domain of the cloned gene are believed to represent the Gm (1) allotypic determinant, whereas the glu-glu-met present in Eu correlates with the Gm (non-1) variant. Thus the cloned gene reported here encodes a polypeptide with the genetic markers Gm (1,17). The Nie heavy chain also carries these markers, yet differs at amino acid number 41 of the C_H3 domain (Nie has arginine as compared to a tryptophan codon for the cloned sequence).

Sequence divergence among three human C_γ genes

We have previously reported the nucleotide sequences of genes encoding C_H regions of human γ2 and γ4 heavy chains (10,11). Our analysis of a $C_{\gamma 1}$ gene allows a

Table 1 Intron and exon lengths in C_γ genes

	length of gene segment (nucleotides)							
C_γ gene	C_H1	C_H1-hinge intron	hinge	hinge-C_H2 intron	C_H2	C_H2-C_H3 intron	C_H3	3' UT
human γ1	294	388	45	118	330	96	321	~130
human γ2	294	392	36	118	327	97	321	~130
human γ4	294	390	36	118	330	97	321	~130
mous γ1	291	356	39	98	321	121	321	93
mous γ2a	291	310	48	107	330	112	321	103
mous γ2b	291	316	66	107	330	112	321	103

The data for the mouse genes are from reference 8. The human γ2 and γ4 numbers come from references 11 and 10, respectively. The lengths of the 3' untranslated (UT) regions in th human genes are determined by homology to the corresponding regions in mouse C_γ genes (see Fig. 5 of reference 10).

comparison of three members of the human C_γ gene family. A summary of the nucleotide sequence comparisons is shown in Table 2. Nucleotide differences in the various noncoding regions are similar, and so values are listed for the total divergence in noncoding DNA. Similarly, each of the C_H exons show similar homologies among the thre genes, and the total observed differences for these exons are given. Hinge exons, on the other hand, show much greater variation than any other gene segment, and these regions are separately compared. Table 2 shows that the level of nucleotide substitution (not including gaps) in noncoding areas is not much greater than the total (silent plus amino acid replacement) seen in the C_H coding regions. Except for areas surrounding the site of polyadenylation of the mRNA (16) and splice junctions (17), the noncoding segments of these genes have no known function. If thes sequences are without any function, they are presumably not subjected to natural selection and are free to diverge. Estimates of the rate of appearance of nucleotide substitutions in unselected noncoding DNA (18) lead us to conclude that approximately 6-8 million years have elapsed since any two of these genes shared an identical sequence. The similar homology levels seen in the three pairwise comparisons make it difficult to determine which two genes shared the most recent

Table 2 Nucleotide sequence comparisons of three human immunoglobulin C_γ genes

genes compared	% nucleotide difference*				
		C_H exons		Hinge exons	
	total noncoding areas	silent	replacement	silent	replacement
γ1 vs. γ2	4.7 (14 gaps)‡	1.6	1.9	2.7	11.1
γ1 vs. γ4	5.4 (18 gaps)	2.3	2.2	2.7	16.7
γ2 vs. γ4	4.6 (4 gaps)	2.0	1.6	3.3	16.7

* This is calculated as (number of substitutions/number of residues compares) x 100. Gaps were not compared.

‡ These were introduced into one or another of the compared sequences to maintain the homology alignment.

common ancestor. However, significantly fewer gaps need to be placed in the noncoding areas of the $C_{\gamma 2}$ and $C_{\gamma 4}$ genes to maintain the homology alignment of the two sequences. This observation along with the determined linkage of these genes (11) suggests that they diverged more recently from each other than from the $C_{\gamma 1}$ gene.

Coding sequence divergence in and near the hinge

The most interesting areas of these genes in evolutionary terms are the hinge exons, which Table 2 indicates are the most divergent gene segments. The differences listed do not reflect the fact that the $C_{\gamma 2}$ and $C_{\gamma 4}$ hinge exons encode three fewer amino acids than the $C_{\gamma 1}$ hinge exon, which codes for 15 residues. The DNA sequence alignment giving maximum homology among the three genes in this exon is shown in Fig. 3. Here we see that distinct nine-nucleotide gaps are placed in the $C_{\gamma 2}$ and $C_{\gamma 4}$ sequences. On either side of these gaps are small coding stretches which are homologous in the three C_γ genes. Every nucleotide substitution indicated in the $C_{\gamma 2}$ and $C_{\gamma 4}$ sequences is in a triplet which encodes an amino acid unique to that hinge region. The combination of nucleotide substitution and insertion/ deletion events leads to quite different coding properties in the hinge exons for the three C_γ genes. Fig. 4 shows the predicted amino acid sequences for the three hinge segments, as well as some contiguous residues in the C_H1 and C_H2 domains. The alignment shows that coding sequence diversity is not limited to the hinge exon itself, but is also

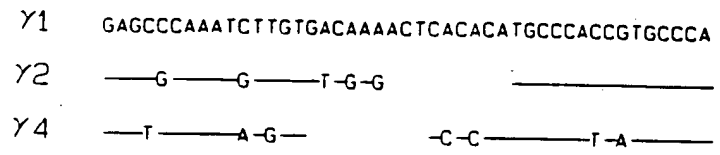
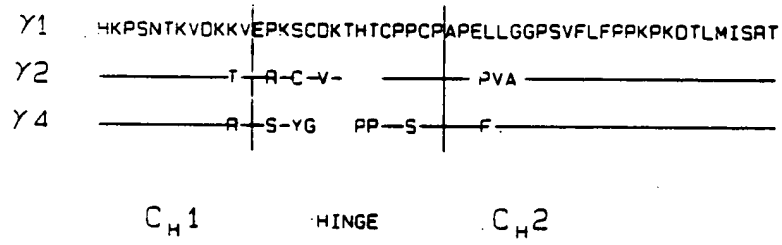


Figure 3. Comparison of hinge exon nucleotide sequences. Solid lines represent identity of the γ2 and γ4 sequences to the γ1 sequence. Where differences occur in the γ2 and γ4 exons, the relevant residues are listed. Gaps are introduced into the γ2 and γ4 listings to maximize homology to the γ1 sequence.

found in areas of the C_H domains which are adjacent to the hinge. Again both base substitution and insertion/deletion events produce coding differences; the latter type of event leads to nucleotides in the C_H2 exon of the $C_\gamma2$ gene being read in a different translational reading frame than their homologous counterparts in the other two genes (see Fig. 2 of ref. 11). Thus although the three genes encode polypeptides which are at least 95% identical over most of their length, amino acid substitutions are clustered in the hinge areas of the proteins. We believe that the high level of divergence in this region exists because natural selection favors the generation of diversity in this part of the molecule. This is not to say that the rate of nucleotide substitution is greater in the hinge than in the more conserved noncoding regions, but rather that substitutions in the hinge area are more rapidly fixed by selection. The nature of the selective advantage offered by hinge variation is not obvious, although it has been suggested that divergent hinges may be responsible for the differences in effector functions carried out by IgG subclasses (3,19,20). If this view is correct, then the generation of new and diverse effector functions may be the selective force which fixes nucleotide changes in the hinge area and the hinge exon itself.



Figur 4. Comparison of amino acid residues in the hinge area of three C_γ polypeptides. Vertical lines separate the hinge residues from those contiguous amino acids which are encoded in the C_H1 and C_H2 exons. Amino acids are listed in the one-letter code. Solid lines represent identity of the γ2 and γ4 sequences to the γ1 sequence. The C_H2 domain of the $C_{\gamma2}$ sequence contains one less amino acid than is found in the other genes.

An unresolved evolutionary issue

Our current picture of human C_γ genes is that they have diverged recently from one another, and that hinge regions have evolved rapidly since that divergence. What is not clear is the nature of the genetic event(s) giving rise to the identical C_γ genes which were the ancestors of the present-day genes. There are two likely alternatives for the generation of two or more identical sequences: (1) a duplication of a single gene sequence, thus producing a gene de novo, and (2) a gene correction process (21) in which all or part of the sequence of one gene is replaced by the sequence of a nonallelic but homologous gene. The latter explanation implies that members of a multigene family do not evolve independently of one another, but rather that genetic information can be exchanged between nonallelic members of a gene cluster. Molecular evidence for the occurrence of such events has been cited for human (22,23) and mouse (24) globin genes and for mouse immunoglobin genes (8). Such evidence consists of the finding of a presumed recombination breakpoint which separates areas of a gene which either were or were not involved in a genetic exchange with another member of the gene family. This breakpoint defines a relatively sharp boundary on either side of which two nonallelic genes share different levels of homology. A boundary of this kind is not found in a comparison of the three human C_γ genes, since except for the extensive divergence found in the hinge region, the nucleotide differences are distributed rather evenly over the length of the genes. If evidence exists for recombination between any two of these nonallelic genes, it is most likely to be found in regions flanking the coding areas that we have characterized.

Thus we are unable to distinguish between the above two alternatives, although we have argued (11) that gene duplication and gene correction are not mutually exclusive concepts. The same kinds of fundamental genetic processes that result in gene duplication can also bring about gene correction. We think it likely that these genetic processes have continued to act on human C_γ genes since the occurrence of the initial duplication event(s). According to this view, our estimated time of divergence of human C_γ genes represents the time elapsed since the most recent correction event. Thus we believe that the human C_γ gene family is probably much older than indicated by the extensive homology shared by its members.

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Attachment No. 3

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Adenovirus-mediated Transfer of the Muscle Glycogen Phosphorylase G ne into Hepatocytes Confers Altered R gulation of Glycogen Metabolism*

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The muscle isozyme of glycogen phosphorylase is potently activated by the allosteric ligand AMP, whereas the liver isozyme is not. In this study we have investigated the metabolic impact of expression of muscle phosphorylase in liver cells. To this end, we constructed a replication-defective, recombinant adenovirus containing the muscle glycogen phosphorylase cDNA (termed AdCMV-MGP) and used this system to infect hepatocytes in culture. AMP-activatable glycogen phosphorylase activity was increased 46-fold 6 days after infection of primary liver cells with AdCMV-MGP. Despite large increases in phosphorylase activity, glycogen levels were only slightly reduced in AdCMV-MGP-infected liver cells compared to uninfected cells or cells infected with wild-type adenovirus. The lack of correlation of phosphorylase activity and glycogen content suggests that the liver cell environment can inhibit the muscle phosphorylase isozyme. This inhibition can be overcome, however, by addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which increases AMP levels by 30-fold and causes a much larger decrease in glycogen levels in AdCMV-MGP-infected cells than in uninfected or wild-type adenovirus-infected controls. CCCP treatment also caused a preferential decrease in glycogen content relative to glucagon treatment in AdCMV-MGP-infected hepatocytes (74% *versus* 11%, respectively), even though the two drugs caused equal increases in phosphorylase *a* activity. Introduction of muscle phosphorylase into hepatocytes therefore confers a capacity for glycogenolytic response to effectors that is not provided by the endogenous liver phosphorylase isozyme. The remarkable efficiency of adenovirus-mediated gene transfer into primary hepatocytes and the demonstration of altered regulation of glycogen metabolism as a consequence of expression of a non-cognate phosphorylase isozyme may hav implications for gene therapy of glycogen storage diseases.

Glycogen is the storage form of the simple sugar glucose, and its presence in mammalian tissues provides a source of metabolic energy for periods of catabolic activity. Digestion of glycogen to yield glucose 1-phosphate (Glc-1-P)[1] is accomplished by the rate-limiting enzyme of glycogenolysis, glycogen phosphorylase. There are three distinct genes encoding glycogen phosphorylases in mammals, termed muscle, liver, and brain after the tissues in which they are preferentially expressed (1–3), that map to separate chromosomes in humans (4, 5) and mice (6). The proteins encoded by the three human glycogen phosphorylase genes are 80–83% identical in primary sequence (1–3, 7). Despite this high level of sequence conservation, the three phosphorylase isoforms have distinctive functional characteristics that are reflected in their different physiological roles. Thus, muscle phosphorylase is acutely regulated not only by phosphorylation (activation) and dephosphorylation (inactivation) at Ser-14, but is also sensitive to a number of allosteric effectors, including the activator AMP, and the inhibitors glucose, glucose 6-phosphate, ATP, and purine nucleotides (1, 8–10). Responsiveness to metabolic intermediates, especially AMP and related molecules, is in keeping with a physiological role for muscle phosphorylase of maintaining a high energy charge in an energy-requiring tissue. Liver phosphorylase, in contrast, is generally less sensitive to allosteric regulation than muscle phosphorylase. The liver enzyme is particularly unresponsive to activation by AMP, consistent with its primary role as a supplier of free glucose for extrahepatic metabolism, a function that might be compromised by inappropriate sensitivity to alterations in AMP levels.

Recently, we have begun to dissect the structural determinants of isozyme-specific allosteric regulation, with particular emphasis on the vastly different responses of the liver and muscle isozymes to AMP. This work has involved detailed *in vitro* kinetic studies on purified proteins (11). Such approaches do not, however, address the functional significance of allosteric regulation in intact cells. In this context, it is interesting to note that the phenotypic consequence of phosphorylase kinase deficiency is different in liver and muscle. Thus, the I-strain mouse, which specifically lacks muscle phosphorylase kinase, has only mildly elevated muscle glyco-

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[1] The abbreviations used are: Glc-1-P, glucose 1-phosphate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CMV, cytomegalovirus; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

gen levels and exhibits no obvious impairment when subjected to a regimen of intense exercise, suggesting that AMP activation of muscle phosphorylase compensates effectively for the lack of covalent activation (12, 13). The *gsd/gsd* rat, in contrast, lacks liver phosphorylase kinase and contains hugely elevated levels of hepatic glycogen, indicating that allosteric activation does not compensate for the phosphorylase kinase deficiency in this tissue (14). These data suggest that introduction of either muscle phophorylase or liver phosphorylase(s) engineered for AMP responsiveness into liver cells might impart altered modes of phosphorylase activation, leading to changes in the regulation of glycogen metabolism in response to physiological signals. In the current study, we have tested this idea by using recombinant adenovirus to transfer the muscle phosphorylase cDNA into liver cells. Our results confirm the hypothesis and show that the recombinant adenovirus system is an extremely efficient means of introducing metabolic regulatory proteins into non-proliferating cells for evaluation of their impact on metabolic flux. This approach may also have implications for studies involving transfer of genes encoding metabolic enzymes into cells and tissues of animals with glycogen storage diseases.

MATERIALS AND METHODS

Cell Isolation and Culture—Hepatocytes were isolated from 24-h fasted adult male Wistar rats (180–225 g) by perfusion of the liver with a collagenase solution as described (15). Cells were plated onto collagen-coated plastic dishes (Corning) at a density of 6×10^4 cells/cm^2 in DMEM (4.5 g/liter glucose; GIBCO) supplemented with 10% fetal bovine serum, 100 nM insulin (Boehringer) and 100 nM dexamethasone (Sigma). After cell attachment (4–5 h), the medium was replaced with DMEM containing 0.2% bovine serum albumin (Sigma), 1 nM insulin, and 10 nM dexamethasone and was subsequently changed on a daily basis.

Preparation of Recombinant Adenovirus and Cell Infection—Recombinant adenovirus containing the cDNA encoding rabbit muscle glycogen phosphorylase (16) was prepared by the strategy outlined in Fig. 1. Briefly, the pACCMVpLpA vector was prepared by insertion of the constitutive cytomegalovirus (CMV) early gene promoter/enhancer, the pUC 18 polylinker, and a fragment of the SV40 genome that includes the small T-antigen intron and the polyadenylation signal, into the previously described pAC vector (17). A 2.56-kilobase pair *NdeI*/*HindIII* fragment of the rabbit muscle glycogen phosphorylase cDNA that includes all of the protein coding region was then inserted into the pACCMVpLpA vector. The resulting plasmid was cotransfected with pJM17 (18) into 293 cells (AdE1A-transformed human embryonic kidney cells; Ref. 19) by calcium phosphate/DNA coprecipitation. pJM17 encodes a full-length adenovirus 5 genome interrupted by the insertion of the bacterial plasmid pBRX at position 3.7 map units, thereby exceeding the packaging limit for adenovirus (18). Homologous recombination between the recombinant pACCMVpLpA plasmid and the pJM17 plasmid in 293 cells generates a genome of packageable size in which the adenovirus early region 1 is replaced by the cloned chimeric gene, rendering the recombinant virus replication defective. The resulting virus was named AdCMV-MGP. The presence of the phosphorylase insert in AdCMV-MGP viral DNA was confirmed by restriction enzyme digestion and Southern blotting using an oligonucleotide (5′-GCGTTGGGGTCTG-GCCTAGGGTATCTT-3′) complementary to the 3′ end of the rabbit muscle phosphorylase cDNA (data not shown). Appropriate viral plaques were expanded into stocks containing $5–50 \times 10^7$ plaque-forming units/ml and stored in DMEM supplemented with 10% fetal bovine serum. Hepatocytes were infected after cell attachment to plates by incubation with stocks of wild-type adenovirus type 5 or the recombinant AdCMV-MGP virus for 1 h at a multiplicity of infection of 5.

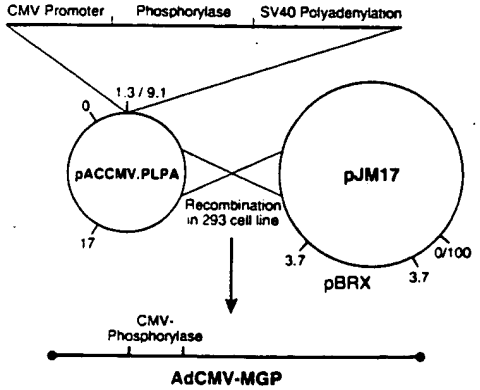

FIG. 1. **Schematic representation of the strategy used for construction of AdCMV-MGP recombinant adenovirus.** The recombinant virus contains a transcription unit consisting of the CMV promoter/enhancer, the rabbit muscle glycogen phosphorylase cDNA, and an SV40 polyadenylation cassette (see "Materials and Methods" for details).

In Situ mRNA Hybridization—The efficiency of infection with AdCMV-MGP was evaluated by performing *in situ* hybridization on AdCMV-MGP-infected and control cells. Cells were harvested 4 days after infection by light trypsinization, washed with phosphate-buffered saline (PBS) and spotted onto silane-coated microscope slides and allowed to settle. Immediately after air-drying, the slides were washed in PBS and treated with fixative solution A (4% paraformaldehyde, 100 mM sodium acetate, pH 6.5) for 10 min, and then with fixative solution B (4% paraformaldehyde, 0.05% glutaraldehyde, and 100 mM sodium tetraborate, pH 9.5) for 10 min. *In situ* hybridization was performed by preparing the rabbit muscle phosphorylase cDNA probe (20) in the presence of digoxigenin-11-dUTP (Boehringer Mannheim). Digoxigenin-labeled probe was then hybridized to cellular muscle phosphorylase mRNA using previously described hybridization and washing conditions (21). Hybridization events were visualized by treating the cells with anti-digoxigenin conjugated to alkaline phosphatase (dilution of 1:400) and subsequent incubation with blocking reagents and alkaline phosphate substrates as prescribed by the manufacturer (Boehringer Mannheim).

Assays of Phosphorylase Activity—Glycogen phosphorylase activities were assayed in hepatocyte extracts by native gel (11, 20) and radioisotopic (11, 22) techniques. For both methods, extracts were prepared by homogenization in a buffer consisting of 10 mM Tris-HCl, pH 7.0, 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. For gel assays, supernatant aliquots were electrophoresed on three identically prepared nondenaturing gels, soaked in buffers containing substrates and various activators, and stained to visualize newly formed glycogen, exactly as described (11, 20). In later gel assay experiments, cleared hepatocyte extracts were treated with purified rabbit muscle phosphorylase kinase as described (11, 23) using 48 μg of supernatant protein and 0.75 μg of enzyme per reaction. For assays monitoring the conversion of [U-^{14}C]glucose 1-phosphate (Glc-1-P) into glycogen (11, 22), the final assay mixture contained 75 mM Glc-1-P, 125 mM KF, and 0.6% glycogen, and labeled Glc-1-P at 0.04 μCi/assay. Glycogen phosphorylase *a* activity was determined by addition of 1 mM caffeine to the assay. In other experiments, AMP activation of phosphorylase *b* was measured by addition of the activator at a final concentration of 5 mM.

Measurements of Glycogen Metabolism—Primary hepatocytes (uninfected or 4 days after infection with wild-type adenovirus or AdCMV-MGP) were preincubated for 4 h in DMEM, 4.5 g/liter glucose, supplemented with 10 mM lactate, 1 mM pyruvate, and 100 nM insulin to maximize glycogen accumulation. Cell monolayers were rinsed with PBS, and subsequent incubations were carried out in DMEM lacking glucose. In addition to control experiments in which cells received no additions, the effects of 1 nM glucagon (Sigma) or 25 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) were studied. Glycogen phosphorylase *a* activity and glycogen content were measured after 20 min or 1 h of incubation, respectively.

Glycogen content was measured by scraping cells into 30% KOH, boiling the homogenate for 15 min, and centrifuging at $5,000 \times g$ for 15 min. The cleared supernatants were spotted onto Whatman 31 ET paper, and glycogen was precipitated by immersing the papers in ice-cold 66% ethanol. After two additional washes in ethanol, the papers were air-dried and incubated with α-glucosidase (Sigma) as described (24). Glycogen-glucose was then measured enzymatically in a Cobas-Bio autoanalyzer with a Glucoquant (Boehringer Mannheim) kit.

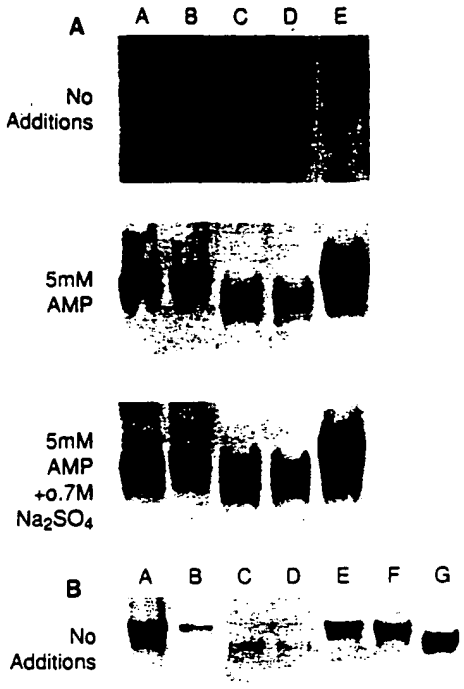

FIG. 2. **Activity gel assays of phosphorylase activities in AdCMV-MGP-infected and control hepatocytes.** *Panel A*, samples were electrophoresed on three identically prepared nondenaturing gels, which were then incubated in a buffer containing phosphorylase substrates and no further additions, 5 mM AMP, or 5 mM AMP + 0.7 M Na_2SO_4, as indicated to the *left* of each *panel*. *Lanes A* and *B* contain 0.15 μg of purified rabbit muscle glycogen phosphorylase *a* and *b*, respectively (Sigma). The following lanes contain extracts (160 μg of total protein/lane) from hepatocytes that were untreated (*lane C*), treated with wild-type adenovirus 5 (*lane D*), or treated with AdCMV-MGP (*lane E*). The migration position of phosphorylase activity in the gel is visualized by staining newly formed glycogen with an iodine solution (11, 20). The extracts in *panel A* were not treated with phosphorylase kinase. *Panel B*, *lanes A* and *B* contain 0.15 μg of purified rabbit muscle glycogen phosphorylase *a* and *b*, respectively. Samples in *lanes C–G* were treated with purified phosphorylase kinase as described under "Materials and Methods" prior to electrophoresis. These lanes contained the following: *lanes C–E*, 20 μg of total protein/lane from hepatocytes that were untreated (*lane C*), treated with wild-type adenovirus 5 (*lane D*), or treated with AdCMV-MGP (*lane E*); *lane F*, 0.15 μg of purified muscle phosphorylase; *lane G*, 0.9 μg of purified liver phosphorylase (11).

RESULTS

Efficiency of Expression Evaluated by in Situ Hybridization—The majority of AdCMV-MGP-infected hepatocytes that were hybridized with digoxigenin-labeled muscle phosphorylase cDNA and treated with anti-digoxigenin-alkaline phosphatase and substrates were clearly stained purple while uninfected cells or cells treated with wild-type adenovirus were uniformly translucent.[2] Counting of cells from 15 different photographed sections of slides revealed that 581 of a total of 675 (86.1%) AdCMV-MGP-infected cells were stained. In contrast, none of a total of 347 control cells (uninfected + wild-type virus-infected) were stained.

Phosphorylase Isoform Expression Assayed with a Native Gel System—Fig. 2*A* shows the results of a native gel activity assay using hepatocyte supernatants prepared in the presence of a phosphatase inhibitor (150 mM KF) and without phosphorylase kinase treatment. Control lanes contain commercially purified (Sigma) rabbit muscle phosphorylase *a* (*lane A*) or *b* (*lane B*). As expected (11, 20), muscle phosph rylase *a* is maximally active under all assay conditions studied, while muscle phosphorylase *b* is potently activated in the presence of AMP alone, approaching the maximal activity induced by incubation in the presence of 5 mM AMP and 0.7 M Na_2SO_4. The latter agent is a potent allosteric activator of all glycogen phosphorylase isoforms (11, 20, 25), and is used in this assay to express total phosphorylase activity. *Lanes C* and *D* contain supernatants prepared from uninfected hepatocytes and from hepatocytes 4 days after treatment with wild-type adenovirus, respectively. In the absence of any effectors it can be seen that the endogenous liver phosphorylase activity migrates as a doublet, probably consisting of the *a* and *b* forms of the enzyme. Incubation of the gel in the presence of 5 mM AMP slightly enhances activity, while strong activation requires the combination of AMP + Na_2SO_4, a result consistent with the relative lack of sensitivity of liver phosphorylase to AMP activation (reviewed in Ref. 1). The phosphorylase activity of primary hepatocytes has a greater mobility in the native gel assay than muscle phosphorylase, due to a distinct predicted isoelectric point (20). Four days after infection with the AdCMV-MGP virus, the migration position and intensity of the phosphorylase activity changes dramatically, as shown in *lane E*. The dominant band now comigrates with the muscle phosphorylase standards, and like purified muscle phosphorylase *b*, is completely activated in the presence of 5 mM AMP alone. These data demonstrate successful expression of the muscle phosphorylase isoform at high levels in primary hepatocytes via the adenovirus system.

To prove that the doublet of endogenous activity seen in Fig. 2*A* was in fact comprised of *a* and *b* forms of liver phosphorylase, we repeated these experiments after treatment of the cleared supernatants with purified phosphorylase kinase. As seen in Fig. 2*B*, kinasing of liver extracts that were untreated or treated with wild-type adenovirus, respectively (*lanes C* and D), resolved the doublet of activity seen in Fig. 2*A* into a single species, consistent with conversion of all endogenous liver phosphorylase activity to the *a* form. As shown in *lane E*, 4 days after treatment with AdCMV-MGP, the phosphorylase kinase-treated extract contains a single band of activity that comigrates with the muscle phosphorylase controls in *lanes A* and *B* and with Sigma muscle phosphorylase *b* that has been treated with phosphorylase kinase (*lane F*). *Lane G* contains native liver phosphorylase, expressed in bacteria and purified to homogeneity as previously described (11), and treated with phosphorylase kinase. The migration position of this activity is identical to that seen in extracts from untreated hepatocytes or hepatocytes treated with wild-type adenovirus (*lanes C* and *D*).

Phosphorylase Activity Measured by Radioisotopic Assay—The native gel activity assay demonstrates clearly a dramatic increase in expression of a phosphorylase activity that comigrates with purified muscle phosphorylase in AdCMV-MGP-infected cells. While these data are useful in defining the isoform that is responsible for changes in activity, they are not quantitative. To address this issue, we performed radioisotopic enzymatic assays (11, 21) of glycogen phosphorylase activities in hepatocyte extracts. Fig. 3 shows the time-dependent changes in phosphorylase activity observed in hepatocytes that were untreated (*panel A*), treated with wild-type adenovirus (*panel B*), or treated with AdCMV-MGP (*panel C*). Consistent with the data in Fig. 2, untreated hepatocytes or hepatocytes treated with wild-type adenovirus contained phosph rylase activity that was only slightly induced (10–33%) by the addition of 5 mM AMP to the assay. Infection with AdCMV-MGP induced an 8–10-fold increase in basal phosphorylase activity relative to the two control hepatocyte

[2] Photographs of treated cells are available upon request.

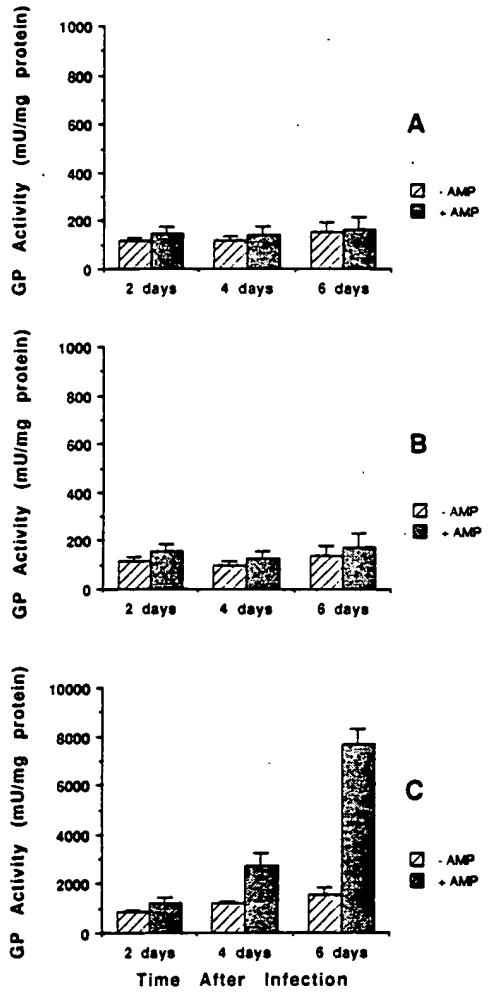

FIG. 3. **Time course of expression of AMP-activated phosphorylase activity.** Hepatocytes were isolated and cultured *in vitro* for the number of days indicated absent virus infection (*panel A*), or after infection with wild-type adenovirus 5 (*panel B*) or AdCMV-MGP (*panel C*). Glycogen phosphorylase activity was then measured in the presence and absence of 5 mM AMP (see *legends* to the *right* of each *panel*) as described under "Materials and Methods." Note the difference in scales when comparing *panel C* to *panels A* and *B*. Data are expressed as the mean ± S.E. for four independent experiments.

preparations (compare *−AMP bar* in *panel C* to *−AMP bar* in *panels A* and *B*). A time-dependent increase in AMP-activatable phophorylase activity was observed in cells infected with AdCMV-MGP. Six days after infection with AdCMV-MGP, phosphorylase in hepatocyte extracts was activated 5-fold by AMP (Fig. 3*C*) and total AMP-activated phosphorylase activity was 46-fold higher than in either the untreated or native virus-infected control cells.

Treatment of Cells with Glucagon and CCCP—The effect of overexpression of muscle glycogen phosphorylase on glycogen metabolism in primary hepatocytes was evaluated by administration of glucagon or CCCP (the latter is a metabolic inhibitor that lowers ATP and increases AMP levels; Ref. 26). The effects of these compounds on glycogen phosphorylase *a* activity (measured in the presence of caffeine to inhibit phosphorylase *b*) are shown in Table I. Infection of hepatocytes with AdCMV-MGP resulted in a 4.7-fold increase in basal phosphorylase *a* activity ($p < 0.001$ c mpared to uninfected or wild-type virus-infected cells). Glucagon and CCCP caused small but statistically significant increases in phosphorylase *a* activity, ranging from 24 to 100% ver control values in the three hepatocyte preparations ($p \leq 0.01$ for all experiments).

TABLE I

Glycogen phosphorylase a activity in hepatocyte extracts

Cells were treated with AdCMV-MGP virus (containing muscle phosphorylase), wild-type adenovirus 5, or vehicle (uninfected) and cultured for 4 days. Glucagon (1 nM) or CCCP (25 μM) was then administered for 20 min and cells harvested for glycogen phosphorylase *a* assay in the presence of caffeine, an inhibitor of phosphorylase *b* (see "Materials and Methods" for details). Data are expressed as milliunits/mg protein and represent the mean ± S.E. of four independent experiments.

Hepatocyte treatment	No addition	Glucagon	CCCP
Uninfected	63 ± 1	94 ± 5[a]	84 ± 4[a]
Wild-type virus	63 ± 2	78 ± 3[a]	81 ± 4[a]
AdCMV-MGP	293 ± 36	515 ± 46[a]	594 ± 66[a]

[a] Statistically significant differences ($p \leq 0.01$) in phosphorylase *a* activity compared to control cells that received no addition. Within each experimental group of hepatocytes, comparison of glucagon *versus* CCCP treatment revealed no significant differences in phosphorylase *a* activities.

TABLE II

Glycogen content in hepatocyte extracts

Cells were treated with AdCMV-MGP, wild-type adenovirus 5, or vehicle (uninfected) and cultured for 4 days. Glucagon (1 nM) or CCCP (25 μM) were then administered for 1 h and cells harvested for assay of glycogen content as described under "Materials and Methods." Data are expressed as μg of glycogen/mg of protein and represent the mean ± S.E. for four independent experiments. Statistically significant changes in glycogen content are indicated by the symbols * ($p \leq 0.01$) and ** ($p < 0.001$) referring to the comparison of glycogen content in the presence of CCCP to glycogen content in the presence of glucagon.

Hepatocyte treatment	No addition	Glucagon	CCCP
Uninfected	388 ± 14	336 ± 15	231 ± 16*
Wild-type virus	412 ± 18	344 ± 16	243 ± 21*
AdCMV-MGP	338 ± 23	300 ± 14	89 ± 16**[a]

[a] $p < 0.001$, refers to the comparison of glycogen content in CCCP-treated AdCMV-MGP-infected cells to glycogen content in either group of CCCP-treated control cells.

The relatively modest effect of glucagon on phosphorylase *a* activity in these experiments is probably due to the preincubation of the cells with high concentrations of insulin (27). There was no significant difference in phosphorylase *a* activity in glucagon- *versus* CCCP-treated cells for any of the experimental groups.

The effect of glucagon and CCCP on glycogen content was measured in parallel hepatocyte cultures. As shown in Table II, infection of hepatocytes with AdCMV-MGP only modestly reduced basal glycogen content relative to uninfected or wild-type virus-infected cells. Treatment of uninfected or native-virus treated hepatocytes with glucagon caused decreases in glycogen content of 14 and 17% relative to basal levels, respectively ($p < 0.05$ for both groups), while the hormone lowered glycogen levels by 11% in cells infected with AdCMV-MGP (not significant). CCCP reduced glycogen content by 40 and 41% in uninfected and wild-type adenovirus-infected hepatocytes relative to cells that received no treatment ($p \leq 0.01$ for both comparisons). In AdCMV-MGP cells the CCCP effect was dramatically enhanced, with a decrease in glycogen content of 74% relative to AdCMV-MGP cells that received no drug; the glycogen levels in CCCP-treated, AdCMV-MGP-infected cells were significantly less than in CCCP-treated uninfected or wild-type virus-infected cells ($p < 0.001$). In addition, glycogen content was significantly less in CCCP-treated *versus* glucagon-treated AdCMV-MGP-infected cells ($p < 0.001$).

DISCUSSION

Much of our current understanding of isozyme diversity, allosteric regulation, and cAMP-mediated functional alteration of enzymes by covalent phosphorylation in mammalian systems originated in classical studies on the family of glycogen phosphorylase proteins (reviewed in Ref. 1). More recently, application of molecular biology (1–6, 11) and crystallography (8–10) to the phosphorylases has yielded a wealth of information about the structural basis for the regulation of enzyme activity. Despite these advances, a number of important questions remain unanswered. One such issue that we have been addressing by site-directed mutagenesis and domain substitution is the structural basis for the dramatic difference in response to AMP allosteric activation exhibited by the liver (poorly activated) and muscle (potently activated) phosphorylase *b* enzymes (11).

With the advent of new gene transfer technologies, a related issue can be addressed, namely the metabolic effect of transferring a tissue-specific isozyme of phosphorylase into a tissue in which it is not normally found. In the current work, we have specifically evaluated the effect of introducing the AMP-activatable muscle glycogen phosphorylase isozyme into primary hepatocytes, which normally express only the AMP-insensitive liver isozyme.

The muscle phosphorylase cDNA was expressed with excellent efficiency in primary hepatocytes using the adenovirus vector/CMV promoter system. *In situ* hybridization experiments indicate that 86% of cells infected with AdCMV-MGP express the muscle phosphorylase mRNA, in keeping with previous reports of high efficiency adenovirus-mediated expression in other cell systems (28). Expression of the transgene was easily detectable 48 h after infection with the AdCMV-MGP virus, and levels of expression increased throughout the 6-day experimental period, with a maximal increase in activity of 46-fold achieved on the 6th day after infection. The possibility that the increased phosphorylase activity is caused by viral effects on endogenous phosphorylase expression is ruled out by two observations: 1) infection with wild-type adenovirus 5 causes no enhancement of phosphorylase activity, and 2) gel activity measurements indicate that the dominant phosphorylase band in extracts from rat hepatocytes infected with AdCMV-MGP comigrates with the rabbit muscle phosphorylase control activities.

The most striking consequence of muscle phosphorylase overexpression in hepatocytes is a strongly enhanced glycogenolytic response to the metabolic inhibitor CCCP. The enhancement is clearly evident when comparing AdCMV-MGP-infected cells to uninfected or native virus-infected controls. It is also obvious when comparing glucagon-treated *versus* CCCP-treated AdCMV-MGP-infected hepatocytes. The most plausible interpretation of the data is that the potent CCCP effect on AdCMV-MGP-infected cells is largely due to AMP allosteric activation of the introduced muscle phosphorylase. In support of this construct, CCCP has been shown to cause an increase in AMP from a basal (untreated) level of 0.1 μmol/g liver to 3 μmol/g liver after only 10 min of incubation (26). A level of 3 μmol/g AMP is roughly equivalent to a concentration of 5 mM, an amount that strongly activates muscle phosphorylase *b in vitro* (29). Furthermore, the level of phosphorylase *a* activity was similar in glucagon- and CCCP-treated hepatocytes, meaning that covalent activation cannot explain the greatly enhanced glycogenolytic effect of CCCP relative to glucagon. The increase in phosphorylase *a* activity in all groups f CCCP-treated hepatocytes is consistent with previous reports (26) and may be due to activation of phosphorylase kinas by Ca^{2+} or nucleotides (30).

An intriguing finding of this study is that increasing phosphorylase *a* activity many-fold by introduction of the muscle phosphorylase isozyme into hepatocytes has little effect on glycogen degradation, except in the presence of CCCP (see Tables I and II). Possible interpretations include the following: 1) intact hepatocytes lack a ligand or factor that is present in muscle tissue and that is required for full expression of the enzymatic activity, and 2) the concentrations of certain allosteric inhibitors are higher in hepatocytes than in muscle cells. Candidate inhibitory ligands could include glucose and UDPglucose. Muscle phosphorylase is inhibited by UDPglucose with a K_i of 0.9 mM (31). The concentration of this ligand in muscle is only 40 μM, but in liver, levels of UDPglucose that could become relevant for inhibition of phosphorylase (0.5 mM) have been reported (32). A suppressor role for the allosteric inhibitor glucose is suggested by the fact that muscle contains the insulin-requiring glucose transporter GLUT-4, while liver contains GLUT-2, which has a high capacity for transport and no insulin requirement. As a consequence of this expression pattern and relative rates of glucose usage in the two tissues, the intracellular concentration of glucose in hepatocytes is usually similar to the circulating level, while free glucose is difficult to detect inside muscle cells. Large increases in AMP concentration, like those induced by CCCP administration, are apparently sufficient to overcome the lack of activating factors or effects of inhibitors. It should be noted that increases in the levels of the phosphorylase substrate P_i could also contribute, since such increases are thought to enhance phosphorylase *a* activity in hepatocytes subjected to anoxia or treated with KCN (33). It will be of interest to determine whether introduction of native liver phosphorylase or liver phosphorylase proteins engineered for AMP activation (11) into liver cells allows greater glycogen degradation under basal conditions or upon glucagon stimulation than observed in the face of muscle phosphorylase overexpression.

Replication-defective adenovirus represents an increasingly popular strategy for gene transfer (28, 34–37). Compared to more commonly used retroviral systems, adenovirus-mediated gene transfer may have several distinct advantages in that expression of foreign genes is not restricted to replicating cells, large DNA inserts are accommodated, and surprisingly persistent expression is maintained (34, 35) despite the fact that the vector does not integrate efficiently into the host cell genome (38). A currently emphasized gene therapy strategy involves the isolation of primary tissue (liver or hematopoetic stem cells, for example), infection of the cells *in vitro* with recombinant retroviruses, and reimplantation of the engineered cells (39, 40). Familial hypercholesterolemia and phenylketonuria are examples of metabolic diseases involving hepatic lesions that may be amenable to this therapeutic approach, since even small systemic increases in the deficient protein (the LDL receptor or phenylalanine hydroxylase, respectively) should be sufficient to cause major improvements in the clinical phenotype (39–41). This approach will not work well for the glycogen storage diseases, however, since the deleterious effects of such syndromes are caused by the overproduction of a cellular intermediate (glycogen), as opposed to th overproduction of a metabolite in the circulation. Therapy for such disorders will likely require a method for efficient introduction of genes into cells in intact organs, even those in which cell division occurs at a low rate. The adenovirus system may be ideally suited f r such *in situ* gene transfer strategies, as indicated by the data described herein, as well as recent experiments by others in which long term

expression of introduced genes has been achieved by introduction of recombinant adenoviruses into whole animals (34, 35). The intense effort devoted to understanding the safety and efficacy of the retroviral vectors in recent years must now be duplicated for the recombinant adenovirus system.

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Basic Science Reports

Adenovirus-Mediated Transfer of a Gene Encoding Human Apolipoprotein A-I Into Normal Mice Increases Circulating High-Density Lipoprotein Cholesterol

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Background In animal models of atherosclerosis, augmentation of circulating high-density lipoprotein (HDL) cholesterol exerts a protective effect against development of fatty streaks and promotes plaque regression.

Methods and Results To investigate the potential of gene transfer to increase HDL cholesterol, a fusion gene encoding human apolipoprotein A-I (apo A-I) under the control of the human cytomegalovirus (CMV) immediate-early promoter was packaged into a recombinant adenovirus (AdCMV apo A-I). BALB/c mice infected with AdCMV apo A-I by intravenous injection accumulate immunoreactive apo A-I in serum; levels 5 days after infection averaged 168 mg/dL. A 35% increase in HDL cholesterol and a 47% increase in total cholesterol were observed in mice infected with AdCMV apo A-I compared with control viruses. Analysis of size-fractionated lipoproteins revealed that human apo A-I is incorporated into murine HDL particles. Expression of human apo A-I declined to <10% of maximum after 12 days and mRNA encoding apo A-I, prevalent 5 days after infection, was undetectable in the livers of infected mice after 12 days.

Conclusions We conclude that adenovirus-mediated transfer of a gene encoding apo A-I produces transient elevations of circulating HDL cholesterol of a magnitude correlated with important physiological effects. These observations suggest the potential for gene-based therapeutic strategies to reduce cardiovascular risk. (***Circulation.* 1994;90:1319-1327.**)

Key Words • atherosclerosis • genes • viruses • lipoproteins • apolipoproteins

Epidemiological data demonstrate an inverse relation between circulating levels of high-density lipoprotein (HDL) cholesterol and the incidence of clinically significant atherosclerosis.[1-4] This relation holds for even small increments of HDL cholesterol, such that each 1-mg/dL increase in HDL cholesterol level is associated with a 2% to 3% decrement in cardiovascular risk.[4] Experimental evidence also supports a protective effect of HDL against atherosclerosis. Cholesterol-fed rabbits treated by infusion of purified homologous HDL are protected against the development of fatty plaques despite unchanged circulating HDL cholesterol levels.[5-7] This association between HDL cholesterol and the incidence of atherosclerotic vascular disease suggests that strategies to increase circulating HDL could have important clinical application. A modest increase in HDL cholesterol has been observed in patients treated with gemfibrozil,[2] an intervention associated with a reduced incidence of cardiac events. Trials intended to specifically assess the effects of intervention to increase HDL cholesterol on the development and progression of atherosclerosis are in progress.[8,9]

HDL appears to exert its antiatherogenic effect by mediating reverse cholesterol transport, in which cholesterol is mobilized from peripheral tissues and transported to the liver.[10-12] The small, high-density, pre-β subspecies of HDL, comprised predominantly of apolipoprotein (apo) A-I and phospholipid, is thought to act as the physiological acceptor for cholesterol in the extracellular matrix of peripheral tissues.[11] Peripheral availability of this "scavenger" particle appears to be regulated by the rates of synthesis, secretion, and catabolism of HDL.[10-12]

Both clinical and experimental data suggest that the principal protein constituent of HDL, apo A-I, mediates the antiatherogenic activity of HDL[1] and that the rate of production of apo A-I is a critical determinant of circulating HDL cholesterol. Families with both heritably deficient[13-16] and enhanced[17] apo A-I levels have been identified and show corresponding alterations in HDL cholesterol. Persons with familial hyperalphalipoproteinemia appear protected from atherosclerosis, while those deficient in apo A-I show accelerated cardiovascular disease. Mice transgenic for a copy of the human apo A-I gene demonstrate accumulation of human apo A-I in serum, increased circulating HDL cholesterol, and resistance to the atherogenic effects of a high cholesterol diet.[18-21] Thus, while the mechanisms regulating the rate of apo A-I synthesis are not clearly defined, genetic factors appear to exert an important effect.[22]

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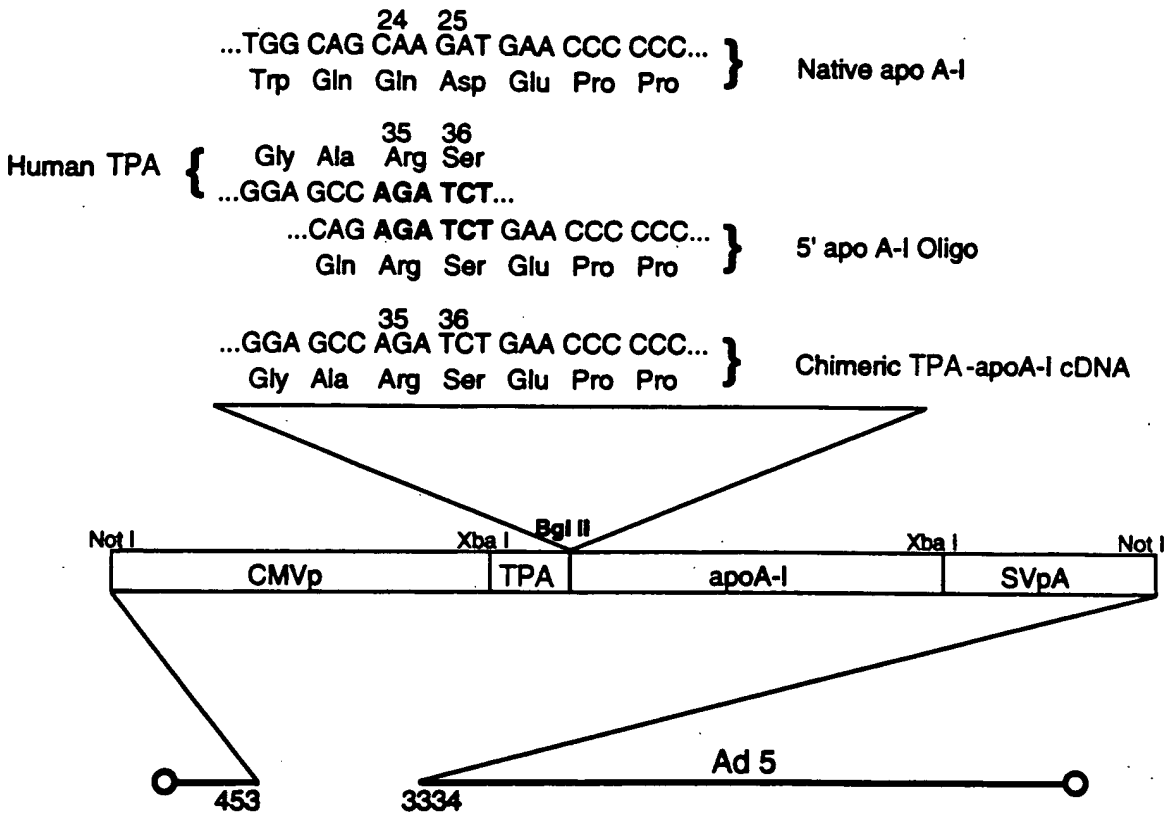

FIG 1. Chart of the construction of AdCMV apolipoprotein (apo) A-I. A fragment of the cDNA encoding human tissue plasminogen activator (TPA) and a partial cDNA encoding the mature human apo A-I protein were ligated in frame using an artificial BglII site (shown in bold type) introduced into the apo A-I sequence during amplification. The resulting chimeric cDNA encodes the mature form of apo A-I behind the secretory signal sequence of human TPA (residue numbers as shown). This cDNA was inserted into pACCMVpLpA between the human cytomegalovirus immediate-early promoter and transcriptional termination and polyadenylation sequences derived from SV40 to form a complete transcriptional unit. The recombinant adenovirus AdCMV apo A-I was generated by homologous recombination between pACCMV apo A-I and pJM17 after transfection into 293 cells.

In view of these observations, somatic cell gene transfer to augment apo A-I expression offers an attractive investigational and potentially therapeutic approach. In this article, we report that normal mice infected with a recombinant adenovirus encoding human apo A-I express high levels of human apo A-I in serum. These animals demonstrate increases in circulating HDL cholesterol similar to those observed in mice transgenic for a copy of the human apo A-I gene and of a magnitude previously associated with a protective effect against the development and/or progression of experimental atherosclerosis.

Methods

Generation of Recombinant Adenoviruses

The recombinant adenovirus (cytomegalovirus, CMV) AdCMV apo A-I was generated by the strategy illustrated in Fig 1. A cDNA encoding mature human apo A-I was obtained by polymerase chain reaction from a human liver cDNA library (generously provided by David Russell) using the oligonucleotide primers 5′:CGGCATTTCTGGCAGAGATCTGAACCCCCCAGA:3′ and 5′:TTTTCTAGAGCCTCACTGGGTGTTGAGCTTCTT:3′. The underlined sequences correspond to positions (relative t the translational start) +55 t +89 and +764 to +788 in the previ usly reported human apo A-I cDNA.[23,24] Sequencing of the cloned amplificati n product demonstrated agreement with the published sequence.

A cDNA fragment encoding the secretory signal peptide from human tissue plasminogen activator (TPA) was obtained by digestion of the plasmid pST, TPA[25] with *Xba* I and *Bgl* II. The amplified partial apo A-I cDNA was digested with *Bgl* II/*Xba* I, and both fragments ligated into *Xba* I cut pACCMV pLpA[26] to produce pACCMV apo A-I. This resulted in an in-frame fusion of sequences encoding the TPA secretory signal sequence with sequences encoding mature human apo A-I. In the resulting fusion protein, the Gln-Asp propeptide cleavage site in native human apo A-I is replaced by the Arg-Ser site from human TPA, introducing an Asp to Ser substitution at the aminoterminus of the mature apo A-I protein. In the plasmid pACCMV apo A-I, this chimeric cDNA is positioned between the human cytomegalovirus immediate-early promoter-enhancer[27] and the polyadenylation transcriptional termination sequences from SV40 to form a complete transcriptional unit.

The pACCMV apo A-I plasmid (10 μg) was cotransfected into 293 cells with 5 μg of pJM17,[28] a plasmid containing a full-length adenovirus 5 genome, by calcium phosphate coprecipitation using a glycerol shock to boost transfection efficiency. Homologous recombination between these plasmids results in the formation of a recombinant adenovirus genome of packageable size in which the CMV apo A-I fusion gene replaces the native adenovirus early region 1. The adenovirus E1A gene product, required for expression of native adenoviral genes, is supplied in *trans* from a copy of early region 1 integrated into th 293 cell genome.[29] Thus, in 293 cells, the recombinant viral gen me is efficiently replicated and packaged into infecti us viral particles.

After transfecti n, m nolayers f 293 cells were overlaid with 0.65% n ble agar in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 2% fetal bovine serum

(FBS, Hyclone). Plaques representing foci of lytic infection became visible 8 to 15 days after transfection, and agar plugs containing the plaques were picked using a sterile Pasteur pipette. Plugs were suspended in 0.5 mL DMEM, subjected t one freeze-thaw cycle, and the resulting suspension (plaque lysate) was used to infect fresh, confluent monolayers of 293 cells. Infected cells were incubated until extensive cytopathic effect was observed.

The identity of recombinant viruses was determined by restriction analysis and Southern blotting of viral DNA prepared from productively infected 293 cells. Infected monolayers were lysed in 0.6% SDS, 10 mmol/L EDTA, pH 8.0, and digested with 20 μg/mL proteinase K for 1 hour at 37°C. High-molecular-weight DNA was precipitated by the addition of 0.25 vol 5 mol/L NaCl and incubation on ice for 16 hours and pelleted by centrifugation at 12 000*g* for 15 minutes at 4°C. DNA was purified from the supernatant by phenol/chloroform extraction and ethanol precipitation. After digestion with appropriate restriction endonucleases, viral DNA was electrophoresed in 1% agarose gels and transfered to nylon membranes (Nytran, Schleicher and Schuell) by capillary blotting. Blots were hybridized with probes labeled with ^{32}P by oligonucleotide-primed synthesis from the parental plasmid DNA. Hybridized blots were imaged using a Molecular Dynamics Phosphorimager and ImageQuant software to demonstrate the presence of the appropriate insert.

The recombinant adenovirus AdRR5, which lacks an inserted gene in the E1 position, was generated from pACRR5[30] and pJM17 in the same manner. The plasmid pACCMV TPA was constructed by ligating the *Xba* I fragment encoding human TPA from pST, TPA[25] into *Xba* I-digested pACMV pLPA, and the resulting plasmid was used to generate the recombinant adenovirus AdCMV TPA. Generation of AdCMV Luc, a recombinant adenovirus encoding firefly luciferase, has been described previously.[31]

Preparation of Purified Viral Stocks

Secondary stocks of the recombinant virus were produced by infection of confluent monolayers of 293 cells grown in 10-cm tissue culture dishes. Monolayers were infected by addition of primary virus stock directly to culture plates. Infected cells were incubated at 37°C until >90% the cells showed cytopathic effect, then lysed by one freeze-thaw cycle before the medium/lysate was collected.

Large-scale production of recombinant adenovirus was performed essentially as described previously[32,33] by infecting confluent monolayers of 293 cells grown in 15-cm tissue culture plates with primary stock at a multiplicity of infection of 0.1 to 1.0. Infected monolayers were lysed with NP40 (final concentration, 0.1%) when >90% of the cells showed cytopathic changes. Virus-containing extracts were centrifuged at 12 000*g* for 10 minutes at 4°C to remove cellular debris. Viral particles were precipitated by the addition of 0.5 vol of 20% polyethylene glycol (PEG) 8000, 2.5 mol/L NaCl, and incubation on ice for 1 hour. Precipitated virus was collected by centrifugation at 12 000*g* for 20 minutes. The resulting pellet was resuspended in 20 mmol/L Tris HCl, pH 8.0, containing CsCl (ρ=1.1 g/mL), layered over a discontinuous (ρ=1.3 to 1.4 g/mL) density gradient, and centrifuged for 2 hours at 20 000 rpm in a Sorvall TH641 rotor at 4°C. Recombinant virus was harvested from the 1.3 to 1.4 interface and desalted by chromatography on Sepharose CL4B in an isot nic saline buffer (10 mmol/L Tris HCl, pH 7.4, 137 mm l/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$). Purified virus eluting in the void volume was collected and, after additi n of sterile bovin serum albumin to a final concentration of 0.1 mg/mL, snap-frozen in liquid N_2 and stored at −80°C until used. The titer of infectious viral particles in purified stocks was determined by plaque assay in m n layers of 293 cells.[33] Purified viral stocks f >10^{10} pfu/mL were routinely obtained.

Infection of Cultured Cells

CV-1 cells were cultured in 10-cm tissue culture dishes in DMEM supplemented with 10% FBS (Hyclone) and infected by the addition of various amounts of virus diluted into 1.5 mL of serum-free media directly to the culture plates. Duplicate 10-cm plates were infected for 1 hour with 10^7, 10^8, and 10^9 pfu AdCMV apo A-I (multiplicities of infection [MOI] of approximately 1, 10, and 100). As a control, duplicate plates were infected with AdCMV TPA at MOI of 10 or were mock-infected with serum-free media alone. After 1 hour of exposure to virus, the infecting media was aspirated and the cells reincubated in DMEM supplemented with 10% FBS. After 24 hours, medium was replaced with 8 mL of serum-free DMEM supplemented with 250 μg/mL penicillin and 50 μg/mL streptomycin for 10 days at 37°C. Aliquots of conditioned media were obtained at various intervals after infection for determination of apo A-I concentration.

Animal Experiments

All procedures involving experimental animals were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee. Female BALB/c mice were anesthetized by intraperitoneal injection of 200 μg of Nembutal. An internal jugular vein was exposed through a combination of sharp and blunt dissection, and 0.1 to 0.25 mL of purified recombinant adenovirus stocks (approximately 1×10^9 pfu/mL) was injected intravenously using a tuberculin syringe under direct visualization. Hemostasis was obtained by direct pressure and the incision closed with wound clips. Animals were allowed to recover on a warming tray before being returned to cages, where they were provided with food and water ad libitum. Samples of tail blood were obtained from reanesthetized animals at varying intervals after infection for determination of circulating apo A-I and cholesterol levels. After 1 to 26 days, animals were killed by intraperitoneal injection of 2 mg of Nembutal and exsanguinated. In addition, livers were harvested from some animals for isolation of nucleic acids or histological analysis.

Immunoprecipitation of Apo A-I

Human apo A-I was immunoprecipitated from medium conditioned by AdCMV apo A-I–infected and control CV-1 cells and from serum obtained from control (AdRR5 infected) and AdCMV apo A-I–infected mice using a commercially available goat anti-human apo A-I antibody (Sigma). Precipitated protein was electrophoresed on SDS–15% polyacrylamide gels and stained with Coomassie blue.

Analysis of RNA in AdCMV Apo A-I–Infected Cells and Liver From Infected Animals

Total cellular RNA was prepared from uninfected and AdCMV apo A-I–infected CV-1 cells (48 hours after infection, MOI approximately 100) using the RNA STAT-60 reagent as directed by the supplier (Tel-Test "B"). Similarly, total RNA was prepared from homogenized liver samples obtained from AdRR5 and AdCMV apo A-I–infected mice. Purified RNA (20 μg) was size-fractionated by electrophoresis in formaldehyde/1% agarose gels, capillary-blotted to nylon membrane (Nytran, Schleister and Scheull), and hybridized against human apo A-I sequences uniformly labeled with ^{32}P by oligonucleotide-primed synthesis. Hybridized blots were imaged using a Molecular Dynamics Phosphorimager and ImageQuant software.

Lipoprotein Fractionation

Pooled serum from groups of uninfected, AdRR5-infected, and AdCMV apo A-I–infected mice was brought t a density of 1.21 g/mL by the additi n of solid KBr, layered over a cushion of 1.21 g/mL KBr, and centrifuged for 10 hours at 35 000 rpm and 4°C in a Sorvall TH641 rotor.[34,35] The lipoprotein fractions were collected from the top of the density buffer and chromat graphed on a Superose 6 column essen-

tially as previously described.[36] Absorbance of the column eluate at 280 nm was monitored continuously, and 0.5- to 1-mL fractions were collected for determination of apo A-I, total protein, cholesterol, and triglyceride concentrations.

Assay Procedures

Apo A-I

Apo A-I concentrations in conditioned medium and serum samples were determined using a commercially available immunoturbidometric assay (Sigma) with minor modifications. Aliquots (5 μL) of each sample were mixed with 50 μL of the antibody reagent (goat anti-human apo A-I) in 96-well, flat-bottom ELISA plates (Corning) and incubated for 15 minutes at room temperature. For determining lower concentrations of apo A-I (after lipoprotein flotation and chromatographic fractionation), 50 μL of sample was mixed with 50 μL of antibody reagent. The absorbance was read at 340 nm on a Molecular Devices Thermomax plate reader and analyzed using Softmax software. All apo A-I determinations were performed in duplicate. Standard curves were constructed from apo A-I standards provided by the kit supplier.

Cholesterol

Cholesterol levels in serum were determined using a commercially available cholesterol oxidase–based assay kit (Sigma) by a modification of the assay protocol suggested by the supplier. Aliquots (5 μL) of serum samples were mixed with 100 μL of the enzyme reagent in 96-well, flat-bottom microtiter plates and incubated at 37°C for 5 minutes. For determining lower concentrations of cholesterol, a 100-μL sample was mixed with 100 μL of reagent. Absorbance was read at 490 nm in the Molecular Devices plate reader and analyzed using Softmax software in comparison to standard curves generated using commercial cholesterol standards. All assays were performed in duplicate.

HDL Cholesterol

The concentration of HDL cholesterol in serum samples was determined in the same assay following selective precipitation of low- and intermediate-density lipoproteins. Aliquots (20 μL) of serum were incubated with 4 μL of phosphotungstic acid in 96-well microtiter plates for 5 minutes at room temperature and precipitated lipoproteins pelleted by centrifugation for 10 minutes at 3000 rpm in a Sorvall RT6000. The resulting supernatant was assayed for cholesterol as described.

Triglycerides

Serum triglycerides were determined using a commercially available enzymatic assay (Sigma). Aliquots (5 μL) of serum were incubated with 50 μL of fresh enzyme reagent for 10 minutes at 37°C in 96-well microtiter plates and the absorbance at 490 nm determined in the plate reader. For lower concentrations of triglyceride, 50 μL of sample was mixed with 50 μL of reagent. All assays were done in duplicate and compared with a standard curve constructed from the commercially supplied triglyceride standards.

Evaluation of Hepatotoxicity

Gamma-glutamyl transpeptidase, aspartate aminotransferase, and serum bilirubin levels were similarly determined using commercially available kits (Sigma) according to protocols provided by the supplier modified only by scaling volumes for microtiter plate assays. Absorbance in these assays was determined using a Molecular Devices microtiter plate reader and Softmax software and quantified by comparison against commercial standards.

Recombinant DNA Techniques

Manipulation of recombinant DNA was performed essentially as. described.[37] Enzymatic reactions were performed under conditions recommended by the suppliers.

Histopathology

Sections of liver obtained from mice at various intervals after infection with AdCMV apo A-I were fixed in 0.25% glutaraldehyde in phosphate buffered saline for 24 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for photomicrography.

Data Analysis

Serum levels of human apo A-I, serum lipid concentrations, and circulating enzyme activities were compared using ANOVA. To determine significance between groups, pairwise post hoc analysis with Scheffé's method was used.[38] For all determinations, significance was assumed at $P<.05$.

Results

Cells Infected With AdCMV Apo A-I Express Immunoreactive Human Apo A-I

To determine whether cells infected with AdCMV apo A-I would synthesize and secrete human apo A-I, CV-1 cells cultured in 100-mm tissue culture dishes were infected with 10^7, 10^8, and 10^9 pfu (corresponding multiplicities of infection of approximately 1, 10, and 100). Northern blotting of total cellular RNA isolated 48 hours after infection demonstrated expression of a single species of RNA (approximately 1.2 kb in size) hybridizing to the human apo A-I probe. RNA from uninfected cells demonstrated no hybridization (Fig 2). Time- and dose-dependent accumulation of immunoreactive human apo A-I was observed in medium conditioned by AdCMV apo A-I–infected cells but not control cells (Fig 3). Cells infected at a multiplicity of 100 secreted immunoreactive apo A-I protein at a rate of approximately 47 $\mu g/10^6$ cells per 24 h over a 10-day period after infection.

AdCMV Apo A-I–Infected Mice Express Human Apo A-I

To determine whether infection of intact animals with AdCMV apo A-I would result in accumulation of human apo A-I protein in serum, 13 BALB/C mice were infected by intravenous injection of purified virus. High levels of human apo A-I were detected in serum from infected mice 1 day (251 ± 103 mg/dL, $n=6$) and 5 days (Table) after infection. Serum from uninfected animals or from animals infected with the irrelevant recombinant adenoviruses AdCMV Luc and AdRR5 demonstrated no immunoreactive material detectable above background (<5 mg/dL), confirming that the endogenous murine protein did not significantly cross-react in the immunoturbidometric assay. Similarly high levels of human apo A-I were observed in C57B/6 mice infected with AdCMV apo A-I (data not shown).

SDS–polyacrylamide gel electrophoresis of protein immunoprecipitated from serum of infected mice and from medium conditioned by AdCMV apo A-I–infected CV-1 cells by goat anti-human apo A-I antiserum revealed a 28-kD band comigrating with the authentic human protein (Fig 4).

While high levels of the recombinant human protein were observed in mice 5 days after infection, levels

FIG 2. Northern blot analysis of RNA isolated from uninfected and AdCMV apolipoprotein (apo) A-I–infected CV-1 cells. Total cellular RNA (20 μg) was size-fractionated by electrophoresis in formaldehyde–1% agarose, transferred to nylon membrane, and probed for human apo A-I sequences with a fragment of the human apo A-I cDNA uniformly labeled with ^{32}P by oligonucleotide-primed synthesis.

declined to <10% of maximal by 12 days after infection and were essentially undetectable at 21 days (data not shown). Northern blotting of RNA isolated from the livers of mice 5, 12, and 26 days after infection with AdCMV apo A-I confirmed that expression of the foreign gene was extinguished at the later times (Fig 5).

Expression of Human Apo A-I Increases HDL Cholesterol

To determine whether expression of human apo A-I produced a significant alteration in circulating lipids, apo A-I, total and HDL cholesterol, and triglycerides were assayed in serum samples obtained from uninfected animals and from mice infected with 1×10^9 pfu AdCMV apo A-I or control viruses 5 days after infection (Table). Total serum cholesterol was approximately 47% greater in AdCMV apo A-I–infected mice than in control mice. HDL cholesterol levels, determined after selective precipitation of other lipoproteins with phosphotungstic acid, were approximately 35% greater in AdCMV apo A-I–infected animals. These results are similar to those previously observed in mice transgenic for a copy of the human apo A-I gene.[18-21]

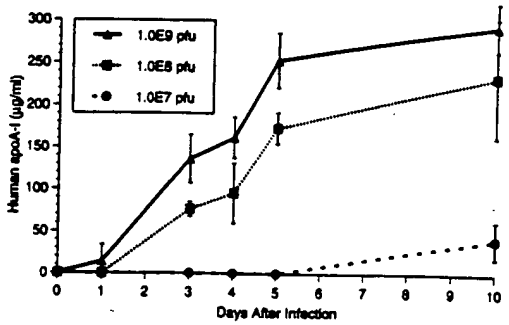

FIG 3. Accumulation of human apolipoprotein (apo) A-I in medium conditioned by CV-1 cells infected with AdCMV apo A-I. CV-1 cells were infected with AdCMV apo A-I at multiplicities of infection of approximately 1, 10, and 100. At the indicated intervals, aliquots of conditioned medium were removed and assayed for apo A-I using a commercial immunoturbidometric assay. Medium conditioned by mock-infected cells or CV-1 cells infected with the control virus AdCMV TPA at a multiplicity of 10 secreted no detectable human apo A-I in the conditioned media at any time point (data not shown).

To further examine alterations in serum lipoproteins induced by overexpression of apo A-I, pooled sera from uninfected mice, from animals infected with AdCMV apo A-I, and from mice infected with AdRR5 was fractionated by KBr density gradient ultracentrifugation. Lipoprotein-containing fractions (density, $\rho<1.21$ g/mL) were further separated by chromatography on Superose 6, and fractions eluting from the column were assayed for protein, cholesterol, and human apo A-I (Fig 6). The human apoprotein coeluted with the predominant cholesterol peak, suggesting incorporation of human apo A-I into HDL particles of appropriate density and size.

Mice infected with AdCMV apo A-I also demonstrated an increase in serum triglycerides (199±81 mg/dL) at 5 days compared with control infected (97±41 mg/mL) or uninfected (125±40 mg/mL) mice, respectively ($P\leq.05$ for both groups compared with AdCMV apo A-I–infected mice). Serum triglycerides in mice infected with either AdCMV Luc or AdRR5 averaged 97±41 mg/dL, a level not significantly different from levels in uninfected animals (mean, 125±40 mg/dL). This suggests that the rise in serum triglycerides is related to overexpression of human apo A-I rather than infection with adenovirus. In general, animals infected with lower doses of AdCMV apo A-I demonstrated smaller increases in serum triglycerides (data not shown). The elution pattern of triglyceride containing lipoproteins from the Superose 6 column was not qualitatively different for serum from AdCMV apo A-I–infected mice in comparison to uninfected or AdRR5 infected mice, although the levels of triglycerides eluting in the very low-density lipoprotein (VLDL) peak were increased.

Infection With AdCMV Apo A-I Is Associated With Transient Hepatic Injury

Infection of experimental animals by adenoviruses has been associated with lymphocytic infiltration of

Human Apolipoprotein A-I and Lipids in Control and Infected Mice

	Human Apo A-I, mg/dL	HDL Ch lesterol, mg/dL	Total Cholesterol, mg/dL	Triglycerides, mg/dL
AdCMV apo A-I–infected mice (n=13)	168±68*†	77±14*†	135±33*†	199±81*†
Control infected mice (n=9)	2±3‡	57±7‡	92±9‡	97±41‡
Uninfected mice (n=10)	1±1	64±8	87±12	125±40

Apo indicates apolipoprotein; HDL, high-density lipoprotein. Values are expressed as mean±SD.
*$P \leq .05$ vs uninfected mice; †$P < .05$ vs control infected mice; ‡P=NS vs uninfected mice.

several target tissues, including liver and lung.[32] To assess whether administration of AdCMV apo A-I to normal mice produced a lymphocytic hepatitis, liver tissue was harvested from animals 5, 12, and 26 days after infection for histological examination. As anticipated from prior observations, a prominent lymphocytic infiltrate was observed in hepatic tissue 5 days after infection (data not shown). Similar infiltrates were observed in liver tissue harvested from AdRR5-infected mice, suggesting a response to the viral vector rather than to the encoded foreign gene. By 12 days, the inflammatory response appeared significantly less, and 26 days after infection, no residual infiltrate was observed. Serum γ-glutamyl transpeptidase (GGT) and bilirubin levels were not increased in AdCMV apo A-I–infected mice 5 days after infection versus AdRR5-infected animals (GGT, 8±1.5 versus 9±0.5 U/mL, P=NS; bilirubin, 0.8±0.3 versus 0.8±0.2 mg/dL, P=NS). Assessment of serum AST levels was confounded by variable degrees of ex vivo hemolysis.

Discussion

The studies described in this report demonstrate that mice infected with a recombinant adenovirus containing a gene encoding human apo A-I efficiently synthesize and secrete the human protein into serum. Protein expressed from the foreign gene is apparently incorporated into physiological HDL particles, and infected

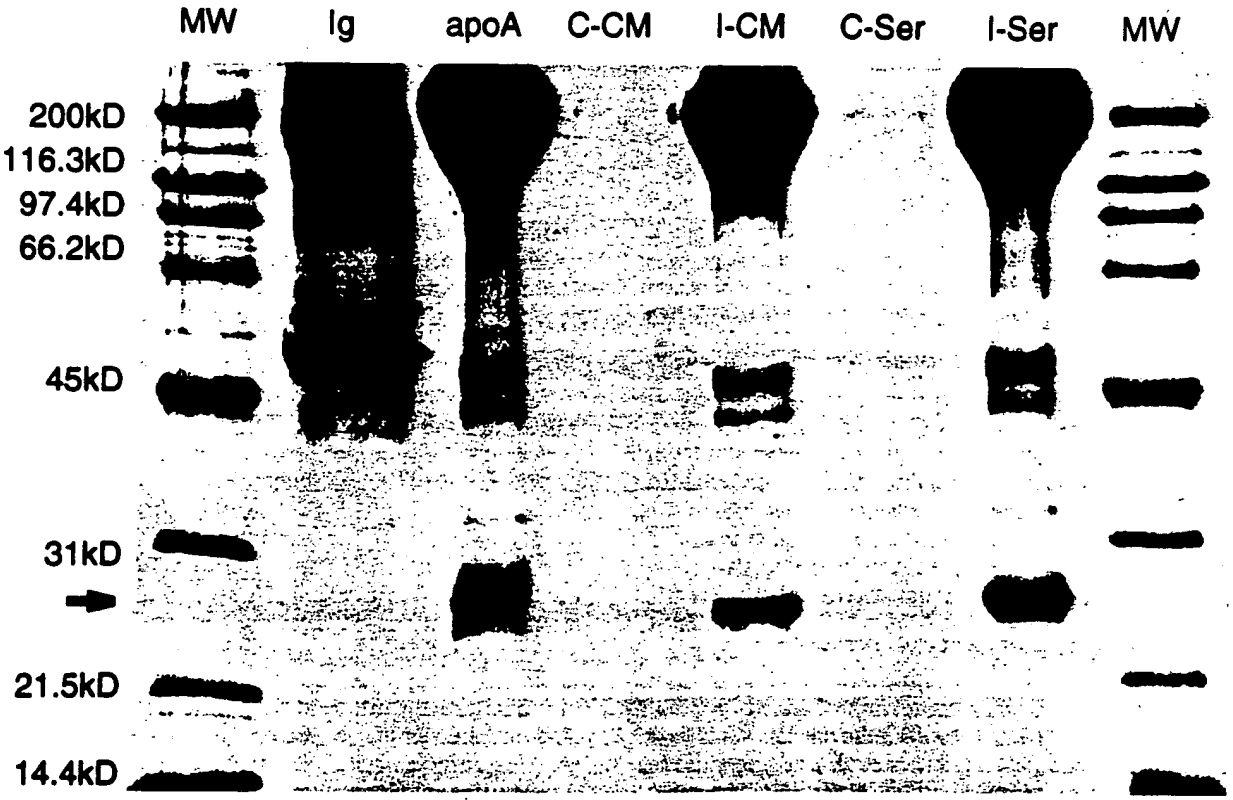

FIG 4. Immunoprecipitation of human apolipoprotein (apo) A-I. Aliquots of conditioned medium or mouse serum wer incubated with goat anti-human apo A-I antiserum. Precipitated protein was collected by centrifugation, separated by electrophoresis in an SDS–15% polyacrylamide gel, and stained with Coomassie blue. Lanes: MW indicates molecular weight standards; Ig, commercial (Sigma) anti-human apo A-I antiserum; apoA, immunoprecipitated purified human apo A-I; C-CM: medium conditioned for 5 days by uninfected CV-1 cells; I-CM, medium conditioned for 5 days by CV-1 cells after infection with AdCMV apo A-I; C-Ser, serum from a mous 5 days after infecti n with AdRR5; I-Ser, serum from a mouse 5 days after infecti n with AdCMV apo A-I. Arrow indicates the position (28 kD) at which human apo A-I migrates.

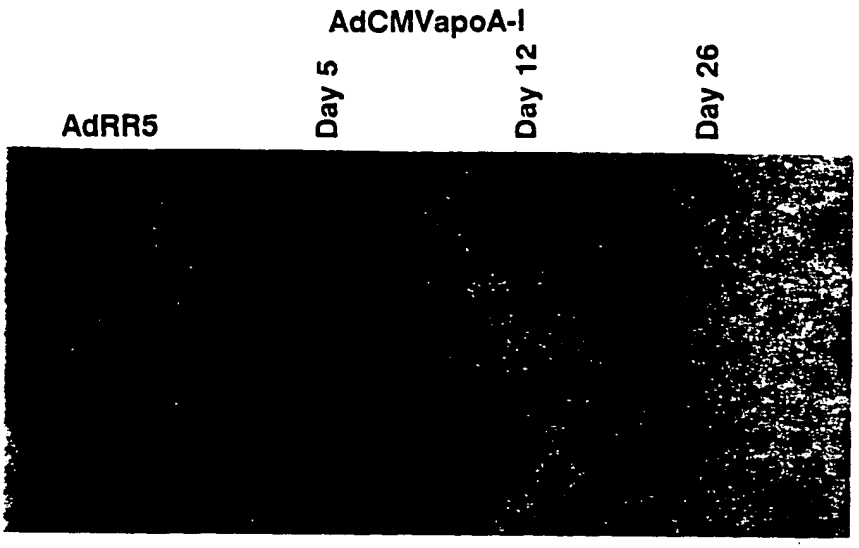

FIG 5. N rthern blot of mouse liver RNA. Total cellular RNA was isolated from the livers of mice 5, 12, or 26 days after infection with AdCMV apolipoprotein (apo) A-I or 5 days after infection with AdRR5, separated by electrophoresis in formaldehyde–1% agaros gels, transferred to nylon membran , and probed for human apo A-I sequences with a fragment of the human apo A-I cDNA uniformily labeled with ^{32}P by oligonucleotide-primed synthesis.

animals demonstrate a significant increase in circulating HDL cholesterol levels. These observations imply that the rate of apo A-I synthesis and secretion from the liver is an important determinant of circulating HDL cholesterol. The magnitude of the increase in HDL cholesterol observed after adenovirus-mediated gene transfer is similar to that observed in animals transgenic for a copy of the human apo A-I gene. The increase in HDL cholesterol observed in these animals, moreover, is large relative to that affording significant protection against the progression of atherosclerosis in both clinical and experimental studies[1-4] and to that associated with a reduction in cardiovascular risk in humans.[2]

Introduction of foreign genes into somatic cells in intact animals has been achieved with a variety of vectors, including recombinant retroviruses, synthetic vectors, and recombinant adenoviruses (reviewed in References 39 through 42), and in some cases expression of cellular proteins or paracrine growth factors has been sufficiently efficient to produce demonstrable physiological effects. In vivo somatic cell gene transfer to produce physiologically meaningful quantities of a serum protein, however, has not been previously demonstrated. The experiments described here demonstrate that adenovirus-mediated gene transfer can result in significant overexpression of a serum protein, highlighting the high efficiency of gene transfer achievable with this vector. While this high efficiency makes adenovirus a very attractive vector for the introduction of foreign genetic material into somatic cells in vivo, our observations also emphasize several important limitations.

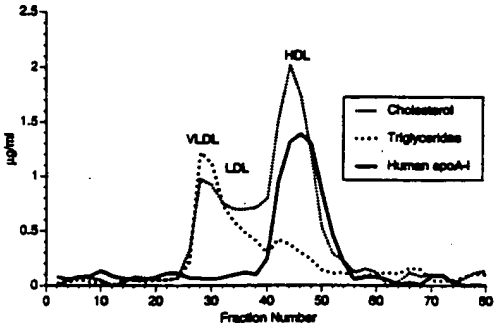

FIG 6. Analysis of density-purified lipoproteins in pooled serum from AdCMV apolipoprotein (apo) A-I–infected mice. Samples of serum obtained from mice 5 days after infection with AdCMV apo A-I were pooled and the lipoprotein containing fraction (ρ<1.21) isolated by KBr density gradient ultracentrifugation. Lipoproteins were further fractionated by chromatography on Superose 6 and fractions luting from the column analyzed for protein, human apo A-I, cholesterol, and triglycerides. Human apo A-I coeluted with the major cholesterol peak in a position corresponding t high-density lipoprotein (HDL). Cholesterol and triglyceride elution profiles for lipoprotein samples prepared from serum fr m uninfected and AdRR5-infected mice wer qualitatively similar (data not shown). VLDL indicates very low-density lipoprotein; LDL, low-density lipoprotein.

Expression of human apo A-I declined rapidly, falling to <10% of peak levels 12 days after infection. In part, this may reflect extinction of expression from the human CMV promoter used in these experiments.[43] Previous experiments[42] have demonstrated that the adenovirus genome is maintained in an episomal state in quiescent somatic cells in vivo and is lost from the livers of infected animals over several weeks,[44] suggesting either that the recombinant genome is unstable in the somatic cells of adult animals or that genetically modified cells are eliminated by host immune surveillance. Prior studies of the stability of foreign gene expression after adenovirus-mediated gene transfer have yielded varying results. Stratford-Perricaudet et al[45] reported expression of β-galactosidase in skeletal muscle for up to 1 year after infection with a recombinant adenovirus. Rosenfeld et al[46] have observed expression of the CFTR gene in pulmonary epithelium for up to 6 weeks after infection, while Herz and Gerard[32] described a rapid decrease in expression of a human low-density lipoprotein LDL receptor gene after intravenous administration of a recombinant adenovirus to mice. While in part these disparities may reflect the end points used to assess expression, they suggest that the stability of foreign gene expression may vary with target tissue, host, or genetic construct. For some potential applications, transient expression of a foreign gene may be sufficient to achieve specific physiological goals. Our observations suggest, however, that strategies to stabilize foreign gene expression in quiescent somatic cells of adult animals will be critical t long-term efficacy after adenovirus-mediated gene transfer.

In mice infected with AdCMV apo A-I, we observed an increase in total cholesterol of a magnitude greater than that accounted for by the observed increase in HDL cholesterol. In addition, mice infected with AdCMV apo A-I but not with AdRR5 of AdCMV Luc demonstrated an unanticipated and significant increase in serum triglyceride levels. On Superose 6 chromatography, the increased triglycerides eluted in the VLDL fraction, suggesting an indirect and previously undescribed effect of overexpression of human apo A-I on VLDL metabolism. These observations contrast with those in mice transgenic for a copy of the human apo A-I gene, in which no significant increase in serum triglycerides were observed.[18,19] Identification of the mechanisms responsible for these alterations in lipoprotein profiles will require an analysis of endogenous murine apolipoproteins and lipoprotein turnover studies. Such might provide insight into unrecognized mechanisms regulating lipoprotein metabolism.

We observed a transient lymphocytic infiltrate in the livers of experimental animals infected with high titers of recombinant adenovirus. Similar inflammatory responses were observed in animals infected with AdCMV apo A-I and control viruses, suggesting that this response is related to the vector rather than the inserted foreign gene. Despite the histopathological abnormalities, serum γ-glutamyl transpeptidase and bilirubin levels were not increased in either AdCMV apo A-I– or AdRR5-infected mice in comparison to uninfected control animals. A similar lymphocytic hepatitis has been observed in mice infected with a recombinant adenovirus encoding the human LDL receptor,[32] and inhalation of human adenovirus 5 has been reported to produce a transient lymphocytic interstitial pneumonitis in rodents in the absence of evidence of viral replication.[47] Whether this immune response results entirely from the administered load of viral antigen or reflects low-level expression of endogenous adenovirus genes by infected cells has not been clearly determined but has important implications for efforts to extend the duration of foreign gene expression after adenovirus-mediated gene transfer and potential implications for the safety of recombinant adenovirus vectors for human gene therapy.[48,49] While to date, adverse effects on the host have been limited to transient inflammatory infiltration of target organs, detailed toxicology studies are unavailable. Moreover, it remains uncertain whether the host immune response to recombinant adenovirus contributes to the extinction of foreign gene expression or whether it will substantially inhibit repeated infection.

Despite the limitations of the current study, these experiments demonstrate the potential for somatic cell gene transfer to augment circulating apolipoprotein levels and specifically to increase circulating HDL levels. While the alterations in lipoprotein levels are transient, the duration of expression should be sufficient for physiological studies of lipoprotein metabolism, alleviating for some purposes the need to generate transgenic animals. This approach may be particularly useful in studies of altered lipoprotein metabolism in (1) larger animals more amenable t studies of vascular biology, (2) species with endogenous lipoprotein profiles more closely resembling those in humans, and (3) species in which generation of transgenic animals is difficult or impractical.

In addition, the potent antiatherogenic effects of HDL make it an attractive target for therapeutic intervention to prevent or retard the progression of atherosclerosis. Both clinical and experimental studies suggest that even minor alterations in HDL cholesterol can exert an important antiatherogenic effect, and the degree of augmentation of HDL cholesterol observed in these experiments has been associated with a protective effect in both experimental animals and humans. Further experiments to determine whether adenovirus-mediated transfer of a gene encoding apo A-I can convey protective effects against the development of vascular disease in well-characterized animal models are warranted, with the goal of evaluating the potential of a gene-based strategy to reduce cardiovascular risk.

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Attachment No. 5

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Medical Sciences

Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice

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ABSTRACT **We have explored the use of adenovirus-mediated gene transfer to transiently elicit production of low density lipoprotein (LDL) receptors in mice. A recombinant adenovirus carrying the human LDL receptor cDNA restored LDL receptor function in receptor-deficient cultured cells. Intravenous injection of recombinant virus acutely lowered plasma cholesterol levels and increased the rate of ^{125}I-labeled LDL clearance from the circulation in normal mice. At 4 days after virus injection, the $t_{1/2}$ of plasma LDL was reduced up to 10-fold. An estimated 90% of the parenchymal cells in liver expressed the adenovirus-transferred genes as judged by immunofluorescence of LDL receptors or by β-galactosidase staining. These results demonstrate that adenovirus-mediated transfer of the LDL receptor gene provides an efficient way of augmenting LDL receptor gene function in the liver over the short term.**

The abiilty to express genes acutely in the liver provides a powerful tool for the study of metabolism and genetic metabolic disorders in intact animals. One example of such a disorder is familial hypercholesterolemia, a frequent human genetic disease that is caused by mutations in the low density lipoprotein (LDL) receptor gene (1–5). Several possible strategies to introduce the LDL receptor and other genes into cells in culture and into tissues *in vivo* have been used in the past (6–15). These experiments have included introduction of the LDL receptor gene into hepatocytes that were successfully returned to the liver (11), and they have also included intravenous administration of a plasmid containing the LDL receptor gene (16).

Recently, adenovirus-mediated gene transfer has been recognized to provide a means of high-efficiency gene transfer into a broad spectrum of eukaryotic cells (12, 17, 18) and into whole animals (6, 7, 19). In a pioneering study, adenovirus-mediated gene transfer was used to cure a rare recessive genetic disorder, ornithine transcarbamylase deficiency, in newborn mice (6). Enzymatic activity expressed from the transferred gene was sustained for up to 15 months. Similarly, the gene for human α_1-antitrypsin has been introduced into the livers of normal rats by intraportal injection (20).

In this study we have investigated the metabolic effect of adenovirus-mediated introduction of the human LDL receptor gene into the normal mouse liver. We have also assessed the efficiency of gene transfer in a quantitative way.

MATERIALS AND METHODS

Preparation f ^{125}I-Labeled LDL (^{125}I-LDL). Human plasma LDL was prepared as described and iodinated by the iodomonochloride method (21). Specific activities of ^{125}I-LDL preparations were 200 and 550 cpm per ng of protein.

Preparati n f Rec mbinant Adenovirus. Recombinant adenoviruses (22) containing the cDNA encoding the human LDL receptor (AdCMV-LDLR) (CMV, cytomegalovirus) (23), β-galactosidase (AdCMV-βGal) (24), and firefly luciferase (AdCMV-Luc) (25) were prepared essentially as described using cotransfection of pACCMVpLpA (26) and pJM17 (27) into 293 cells (28).

Large Scale Preparation of Recombinant Adenovirus. Large scale production of recombinant adenovirus was performed in 293 cells grown either in 15-cm culture dishes or in suspension using Joklik's calcium-free minimum essential medium (GIBCO) supplemented with 10% fetal calf serum. Infected cells were lysed 48 hr postinfection with Dulbecco's phosphate-buffered saline (PBS) (GIBCO) containing 1 mM $MgCl_2$ and 0.1% Nonidet P-40. Virus-containing extracts were centrifuged at 12,000 × *g* for 10 min to remove debris before precipitation of the virus particles by addition of 0.5 vol of 20% polyethylene glycol (PEG) 8000/2.5 M NaCl and incubation on ice for 1 hr. Virus was collected by centrifugation at 12,000 × *g* for 10 min, resuspended in isotonic saline (135 mM NaCl/5 mM KCl/1 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4), and dialyzed against the same buffer overnight before sterilization through a 0.22-μm filter. Alternatively, PEG-precipitated virus was further purified by CsCl density centrifugation essentially as described (29). Equivalent results were obtained with both methods of preparing virus.

^{125}I-LDL Degradation in ldlA7 Cells. Chinese hamster ovary (CHO) ldlA7 cells (30) in six-well dishes were infected with the indicated amount of recombinant virus at densities of 6×10^5 and 1.7×10^6 cells per well in 1 ml of Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum. ^{125}I-LDL (4 μg/ml) in DMEM without glutamine containing 0.2% bovine serum albumin was added to the cells 60 hr after infection and incubation was continued for 6 hr. The appearance of ^{125}I-LDL degradation products in the medium was determined by measuring trichloroacetic acid-soluble radioactivity in the medium as described (21).

Immunohistochemistry. Frozen liver sections were analyzed by immunocytochemistry using polyclonal (31) and monoclonal (32) antibodies as described (31).

***Escherichia coli* β-Galactosidase and Luciferase Assays.** For β-galactosidase staining, frozen sections were fixed at room temperature in 0.5% glutaraldehyde freshly prepared in PBS for 15 min and extensively washed in PBS. β-Galactosidase activity was detected by immersing the sections into 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining solution [35 mM $K_4Fe(CN)_6$/35 mM $K_3Fe(CN)_6$/1 mM $MgCl_2$/1 mg of X-Gal per ml (GIBCO/BRL)] for 15 hr at 37°C. Sections were lightly counterstained with eosin. The enzymatic activity of firefly luciferase was determined as described by deWet *et al.* (25).

Animal Procedures. Mice used in this study were either purchased from Harlan (BALB/c, C57BL/6) or bred in house (outbred animals) and fed ad libitum throughout the course of the experiments. Prior to virus inj ctions and turnover studies, animals were anesthetized by intraperitoneal sodium

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Abbreviations: LDL, low density lipoprotein; CMV, cytomegalovirus; pfu, plaque-forming units.

pentobarbital (Nembutal) injection (100 μg per g of body weight). The external jugular vein was laid open by a skin incision and the indicated virus or ^{125}I-LDL was slowly (over ≈30 s) injected in a total vol of 250 μl. The wound was closed by stapling.

^{125}I-LDL in 10 mM Tris·HCl/140 mM NaCl, pH 7.5, containing 0.2% bovine serum albumin was injected 4 days after virus administration into the opposite jugular vein or into a femoral vein. Blood (50–100 μl) was obtained at the indicated intervals by retroorbital puncture from the anesthetized animals and collected into heparin-treated Pasteur pipettes. ^{125}I-LDL levels in plasma were determined by measuring trichloroacetic acid-precipitable radioactivity of a 20-μl plasma sample.

For determination of ^{125}I-LDL uptake into the different tissues, animals were killed 20 min after injection of the label. Indicated organs were removed and homogenized in PBS, and radioactivity was determined in a sample of the homogenate.

Recombinant DNA Techniques and Protein Determinations. DNA manipulations were performed essentially as described in Sambrook *et al.* (33). Protein concentrations were determined by the method of Lowry *et al.* (34). Cholesterol concentrations were determined by the cholesterol oxidase method (Boehringer Mannheim).

RESULTS

Virally Transferred LDL Receptor Is Functional in Cultured Cells. To quantify functional activity of AdCMV-LDLR, we determined the potency of the recombinant virus to confer the ability to degrade ^{125}I-LDL on CHO cells that carry a defect in their endogenous LDL receptor gene (CHO ldlA7 cells) (30). Fig. 1 shows that the amount of ^{125}I-LDL that is degraded by these cells increases in a linear fashion with the number of plaque-forming units (pfu) of AdCMV-LDLR that had been added to the culture dish. The addition of an equivalent number of AdCMV-βGal virus did not enhance degradation of the added ^{125}I-labeled ligand. The total amount of human LDL receptor protein produced by 293 cells 20 hr after infection with AdCMV-LDLR was also dependent on the amount of virus used to infect the cells (Fig. 1 *Inset*).

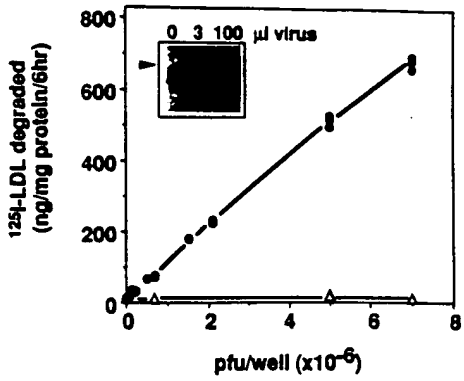

FIG. 1. AdCMV-LDLR leads to a dose-dependent increase of ^{125}I-LDL degradation in LDL receptor-defective CHO cells. LDL receptor-defective CHO ldlA7 cells were infected with the indicated number of AdCMV-LDLR (●) or AdCMV-βGal (△) as described. Degradation of ^{125}I-LDL (4 μg/ml) was measured 60 hr postinfection and normalized to the amount of protein present in each dish. Determinations were performed in triplicate and all individual data points are plotted. Some points are not resolved, as experimental variation was very small. (*Inset*) Western blot of ≈50 μg of 293 cell protein with a monoclonal antibody directed against the human LDL receptor 20 hr after mock infection (0 μl) or after infection with 3 or 100 μl of primary virus stock. Monoclonal antibody bound to LDL receptor was detected with ^{125}I-labeled rabbit anti-mouse IgG (32).

To quantify the efficiency of adenovirus-mediated gen transfer to different tissues *in vivo*, we injected ≈2 × 10^9 pfu of AdCMV-Luc into the external jugular vein of five mice. Animals were sacrificed 4 days after the injection and luciferase activity was determined in the individual tissues (Fig. 2*A*). Low luciferase activity was detected in all of the tissues examined including skeletal muscle and heart, organs that had previously been shown to be target tissues for intravenously injected recombinant adenovirus (36). However, >99% of the total enzyme activity recovered from the

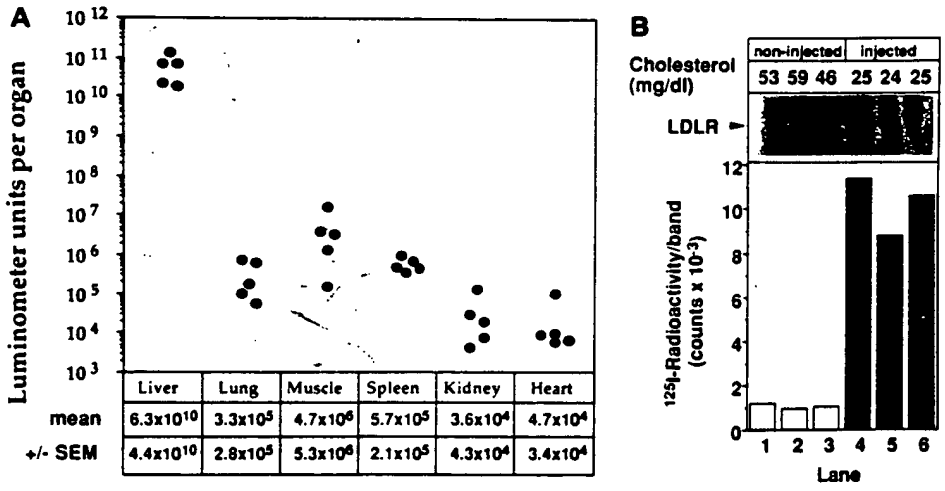

FIG. 2. (*A*) Tissue distribution of luciferase activity in AdCMV-Luc-injected animals: Preferential targeting of adenovirus to the liver. Five outbred hybrid male mice (≈6 months old; 35–45 g) were injected with ≈2 × 10^9 pfu of AdCMV-Luc into the external jugular vein. Luciferase activity in homogenates of the individual organs was determined 4 days after injection of the virus. Activities are expressed as luminometer units per total organ with the exception of muscle, where activity is expressed on a per g basis. Note that enzymatic activity is expressed on a logarithmic scale. Lower detection limit in this experiment was ≈2 × 10^3 units. (*B*) Intravenous injection of AdCMV-LDLR into normal mice leads to LDL receptor overexpression in liver. Six female C57BL/6 mice (12 weeks old; 20–25 g) were either not injected (lanes 1–3) or injected (lanes 4–6) with 2 × 10^9 pfu of AdCMV-LDLR. Four days after injection, the animals were sacrificed, and liver membranes prepared from each animal as described (35) were subjected to Western blotting (250 μg of protein per lane). Migration of the LDL receptor (LDLR) is indicated by the arrowhead. Total radioactivity present in each band was quantified by scanning the blot for 4 hr on an Ambis radioanalytic imaging system. Background (≈200 counts for each sample) was measured in a representative area of the blot and subtracted from the total counts to give the values shown.

injected mice was found in the liver. No luciferase activity was expressed in uninjected animals, which had uniform backgr und levels regardless of the tissue examined (data not shown).

In Viv **Expressi n of Transferred Human LDL Recept r.** To assess whether the recombinant virus was efficient in causing a physiologically relevant increase of LDL receptor activity in the liver, the primary site of lipoprotein catabolism (1), we injected three C57BL/6 female mice with $\approx 2 \times 10^9$ pfu of AdCMV-LDLR into the external jugular vein. Four days later, hepatic expression of the transferred LDL receptor was evaluated by Western blotting. Expression of LDL receptors in the livers of virus-injected animals was increased ≈10-fold over that observed in uninjected control mice (Fig. 2*B*), assuming equal reactivity of the antibody with human and mouse receptors. Virus-injected animals also had significantly lower plasma cholesterol levels when compared with noninjected controls, indicating that the LDL receptor cDNA contained in the virus was transcribed and translated into biologically functional receptors. We next sought to determine the expression pattern of the virally transferred genes for human LDL receptor and *E. coli* β-galactosidas in the livers of injected mice. For this purpose, liver sections of animals that had been injected with either AdCMV-LDLR or AdCMV-βGal were examined by immunofluorescence analysis (Fig. 3 *A–D*) or by histochemical staining for β-galactosidase activity (Fig. 3 *E* and *F*). LDL receptor was expressed only in the livers of animals that had been injected with AdCMV-LDLR (Fig. 3 *A*, *B*, and *D*) and was absent in animals that had been injected with AdCMV-βGal (Fig. 3*C*). Likewise, a similarly high percentage of liver cells of animals that had been injected with the adenovirus construct carrying the β-galactosidase gene were reactive upon histochemical examination for this enzyme (Fig. 3*E*), while β-galactosidase activity was completely absent from the livers of mice injected with AdCMV-LDLR (Fig. 3*F*).

Increased Catabolic Rate of ^{125}I-LDL. To further quantitate the effect of the virus-mediated transfer of exogenous LDL receptor cDNA, we examined the rate of ^{125}I-LDL turnover and steady-state cholesterol levels in animals injected with

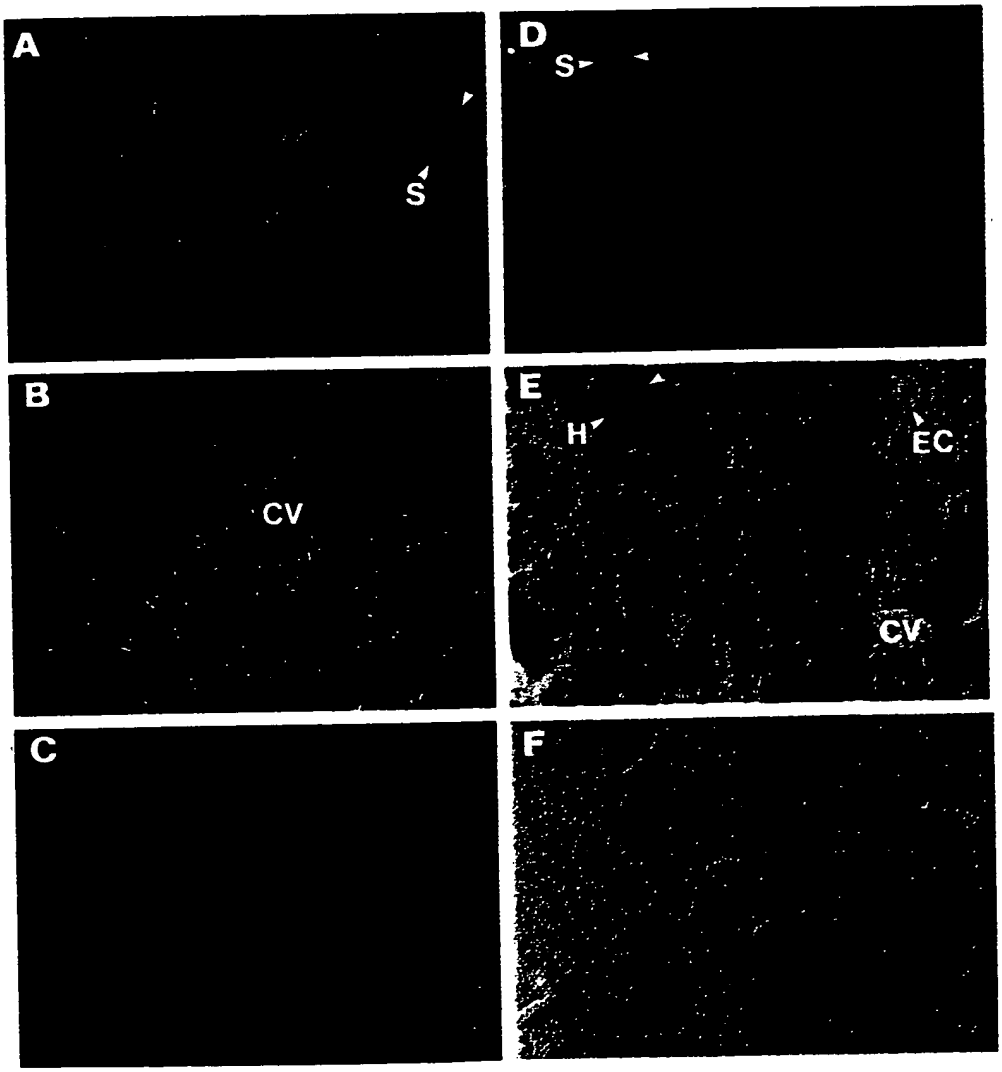

FIG. 3. Immunohistochemical analysis of human LDL receptor expression and histochemical detecti n of β-galactosidase activity in the livers of mice injected with AdCMV-LDLR or AdCMV-βGal. Female BALB/c mice (≈12 weeks old; 20–25 g) were injected with either 3.5 $\times 10^9$ (*A*, *D*, and *F*) or 2×10^9 (*B*) pfu of AdCMV-LDLR or with 2×10^9 (*C* and *E*) pfu of AdCMV-βGal. Expression of LDL receptor in livers of animals was detected with either a polyclonal rabbit IgG (*A–C*) or a mouse monoclonal IgG (*D*) that reacts specifically with the human receptor. Specific staining is only present in the livers of animals injected with AdCMV-LDLR and is absent in the AdCMV-βGal-injected mouse. Up t an estimated 90% of the liver cells are expressing human LDL receptor, which shows the typical polarized expression pattern (37). Sinusoids (S) (*A* and *D*) that have been sectioned along the longitudinal axis are indicated by arrowheads. Expression of β-galactosidase activity (*E*) is f und predominantly in the nuclei of hepatocytes (H) that are arranged in a typical columnar array (arrowheads in *E*). Nuclei of endothelial cells (EC) are stained less intensely. No β-galactosidase activity is found in mice injected with AdCMV-LDLR (*F*). CV, central vein. (×28.)

AdCMV-LDLR versus mice that had received AdCMV-βGal. Fig. 4 shows the results of four separate clearance studies performed with two different virus preparations. ^{125}I-LDL was removed from plasma slowly by the control animals injected with AdCMV-βGal and the clearance rate ($t_{1/2}$, ≈5 hr) was indistinguishable from that previously obs rved in normal mice (38). In contrast, AdCMV-LDLR significantly accelerated removal of the radiolabeled ligand from the circulation of the animals. As a rule, the animals that cleared the ^{125}I-labeled ligand most efficiently also had the lowest steady-state plasma cholesterol levels 4 days after virus administration (Fig. 4 and Table 1). The rate of ^{125}I-LDL clearance was dose dependent and proportional to the amount of pfu of AdCMV-LDLR that had been injected into the mice and was up to 10-fold greater than the rate observed in either normal (38) or AdCMV-βGal-injected animals (this study).

The liver was the only organ in AdCMV-LDLR-injected mice that showed a significant increase in ^{125}I-LDL radioactivity versus control animals when the absolute tissue uptake of the labeled ligand was measured 20 min after injection (Table 1). Up to 45% of the injected dose was recovered in the livers of AdCMV-LDLR-injected mice compared to 13–15% that had accumulated in the controls. Thus, even without compensating for nonspecific trapping of the tracer, significantly more ^{125}I-LDL was taken up into the livers of AdCMV-LDLR-injected animals, in agreement with the results obtained for AdCMV-Luc (Fig. 2*A*).

DISCUSSION

We have explored the feasibility of using replication-defective adenovirus particles to transfer the LDL receptor gene into the liver. Following a single peripheral intravenous injection, a high proportion of hepatic parenchymal cells

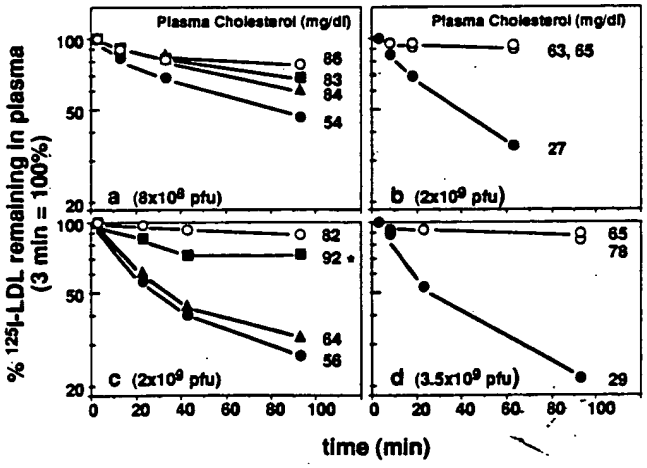

FIG. 4. ^{125}I-LDL clearance from plasma is accelerated in mice injected with AdCMV-LDLR. Female BALB/c mice (≈12 weeks old; 20–25 g) were injected with the indicated amount of AdCMV-LDLR (solid symbols) or with a fixed amount of AdCMV-βGal (2×10^9 pfu; open circles). Four days after virus administration, animals were injected with 15 μg of ^{125}I-LDL. Blood samples were analyzed at the indicated times as described and the radioactivity remaining in plasma was plotted as a percentage of the activity present 3 min after injecti n of the labeled ligand. Four separate experiments were performed. Individual clearance curves for each animal are shown. Steady-state plasma cholesterol levels (mg/dl) of each animal are indicated next t the last time point of the clearance curve. Asterisk den tes a mouse that sh wed shock sympt ms during the clearance experiment and died shortly after the last time point was taken. This animal ceased to clear ^{125}I-LDL between 40 and 90 min. Hepatic circulation was presumably shut d wn as a result of circulatory sh ck.

Table 1. Tissue uptake of ^{125}I-LDL 20 min after injection

	% of injected dose					
	LDLR			β-Gal		
Liver	44.6	44.4	21.9	12.9	15.2	14.7
Kidney	1.1	2.2	2.5	2.2	3.4	2.9
Lung	0.9	1.4	1.8	1.7	1.7	1.4
Spleen	0.5	0.6	0.7	1.1	1.2	1.1
Heart	0.3	0.7	0.7	1.2	1.0	0.9
Plasma cholesterol, mg/dl	42	63	84	81	102	86

Female BALB/c mice (12 weeks old; 20–25 g) were injected with either 2×10^9 pfu of AdCMV-LDLR or the same amount of AdCMV-βGal. Four days after virus administration, the animals were injected with 7.5 μg of ^{125}I-LDL and killed 20 min later. Organs were quickly removed and homogenized in PBS; radioactivity was determined in a sample of the homogenate. Total radioactivity recovered from the individual organs is shown as a percentage of injected dose (4×10^6 dpm). No correction has been made for nonspecific trapping of the ^{125}I-labeled tracer. Plasma cholesterol levels of individual animals are shown in the bottom line.

were infected with the recombinant virus and efficiently expressed foreign genes were harbored within the adenovirus genome. Expression of the human LDL receptor gene resulted in plasma cholesterol levels that were lower than those measured in animals that had been injected with a virus carrying the β-galactosidase gene. Likewise, clearance of ^{125}I-LDL from the circulation was up to 10-fold accelerated in mice injected with AdCMV-LDLR. This is consistent with the ≈10-fold increase of LDL receptor immunoreactivity in the livers of injected mice. Adenovirus-mediated gene transfer therefore can provide strong transient expression of an exogenous LDL receptor gene in the liver, predominantly in the hepatocytes. The liver is the primary target after peripheral intravenous injection of recombinant adenovirus as shown quantitatively in this study and was previously observed by Stratford-Perricaudet *et al.* (36).

We have not yet explored other important questions that are essential to evaluate the potential suitability of the adenovirus system for the treatment of familial hypercholesterolemia or other inborn errors of metabolism that manifest themselves in the liver. These include the choice of the promoter driving the exogenous gene, safety concerns connected with the use of a human virus (20), and the efficiency of virus-mediated gene transfer in patients or animals expressing neutralizing antibodies to adenovirus. It is also currently not clear for how long the adenovirus genome will persist in various tissues and whether persistence of gene expression will be longer in tissues that turn over slowly versus those that regenerate more rapidly (35).

A pathological pneumonia-like response after intranasal inoculation of mice with type 5 adenovirus independent of viral replication has been reported (40). In our study, we have also observed variable degrees of lymphocytic infiltrations in the livers of virus-injected mice (data not shown). The extent of the inflammatory reaction appeared to be proportional to the amount of administered virus and was paralleled by an increase of liver marker enzymes in the plasma. To assess the potential therapeutic use of adenoviral LDL receptor gene transfer into the liver, further investigation of cytotoxic side effects caused by the virus will be required.

In the present experiments, the LDL receptor cDNA was driven by the strong CMV promoter. Overexpression of LDL receptors does n t appear to have any obvious harmful effects in transgenic mice (36) and usage of strong endogenous promoters (like the apolipoprotein AI promoter) might be preferable and could potentially improve long-term expression of the transferred gene. This questi n will have to be addressed in the only animal model available for this [illegible] t date, the Watanabe heritable hyperlipidemic rabbit (39), or

possibly in mice in which the LDL receptor gene has been eliminated by targeted disruption.

Irrespective of whether it will prove to be a feasible approach for somatic gene therapy adenovirus-mediated gene transfer is a powerful method that allows at least transiently a high level of expression of a functional foreign gene in the liver of an intact animal. In many cases this method may obviate the need to create transgenic animals.

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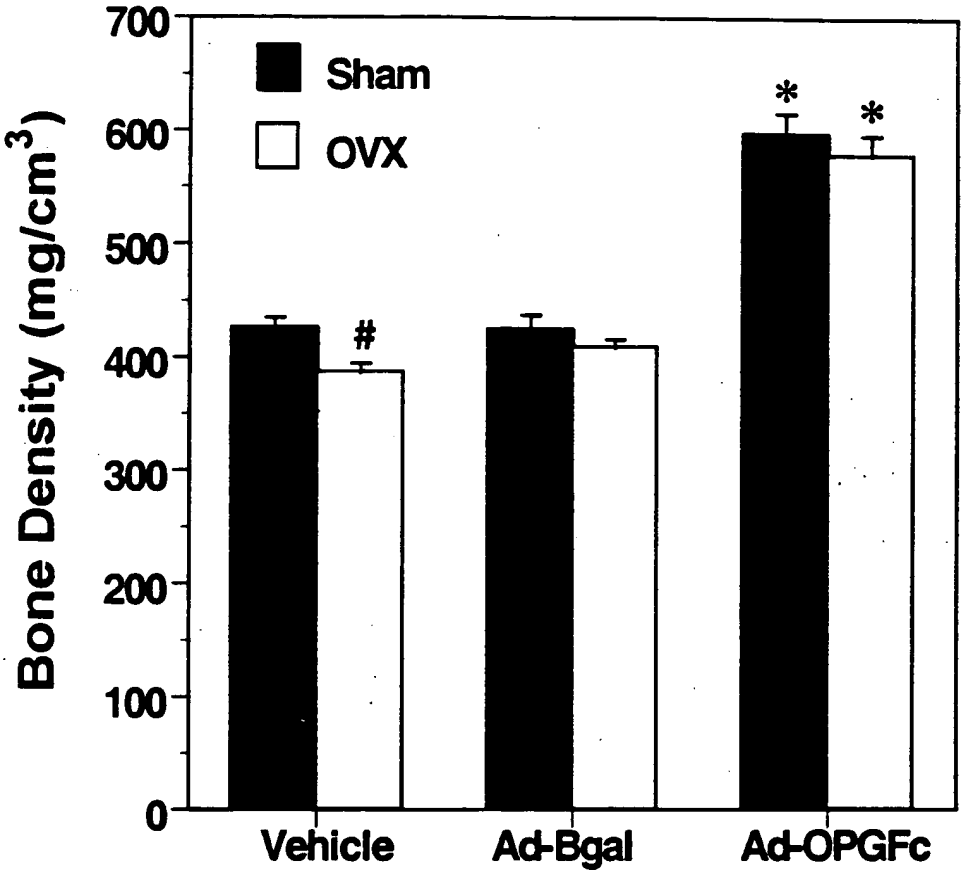
ATTACHMENT NO. 6

BONE MINERAL DENSITY IN THE TIBIAL METAPHYSIS
OF SHAM-OPERATED AND OVARIECTOMIZED (OVX) MICE

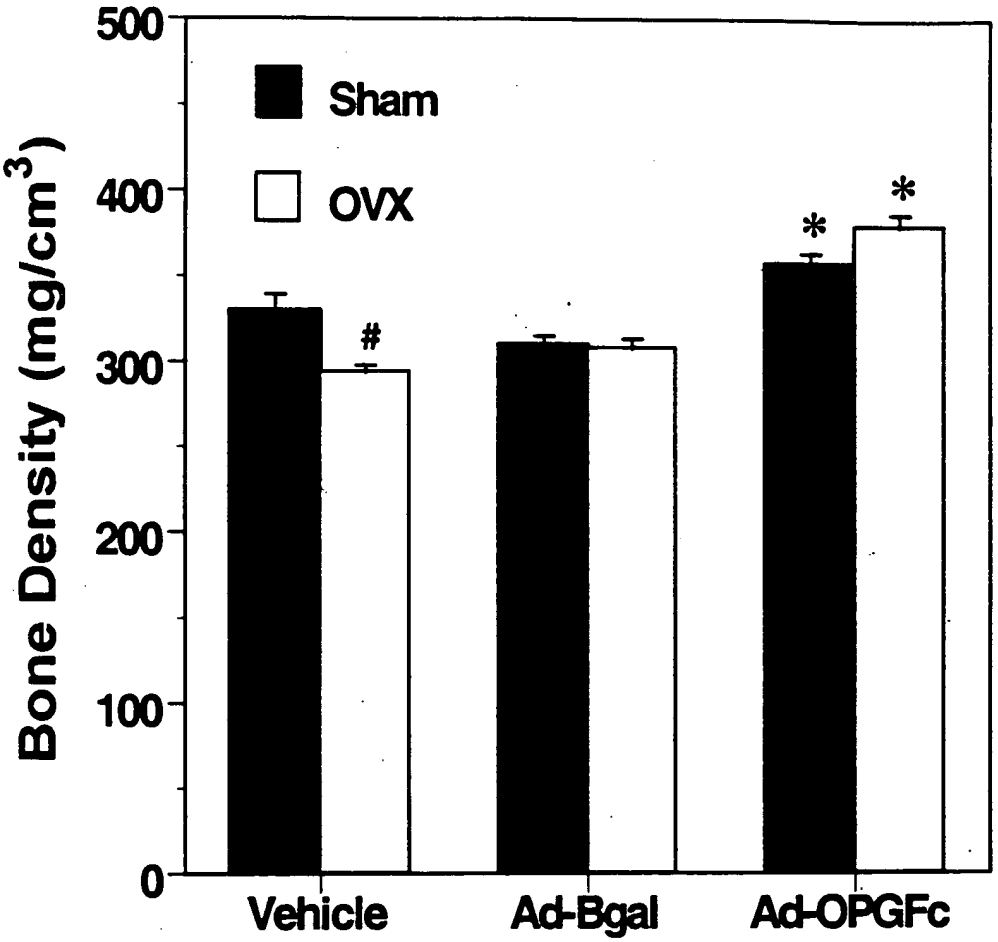


Ovariectomized mice are significantly different from sham-operated mice receiving the same treatment ($p < 0.05$)

* Ovariectomized and sham-operated mice receiving Ad-OPGFc are significantly different from sham-operated mice receiving vehicle ($p < 0.001$)

ATTACHMENT NO. 7

BONE MINERAL DENSITY IN THE FIFTH LUMBAR VERTEBRA OF SHAM OPERATED AND OVARIECTOMIZED (OVX) MICE



Ovariectomized mice are significantly different from sham-operated mice receiving the same treatment, ($p < 0.05$)

* Ovariectomized and sham-operated mice receiving Ad-OPGFc are significantly different from sham-operated mice receiving vehicle ($p < 0.001$)